

**The role of the signaling mediator Smad1 in BMP2-dependent  
osteochondrogenic development in mesenchymal progenitors  
(C3H10T1/2)**

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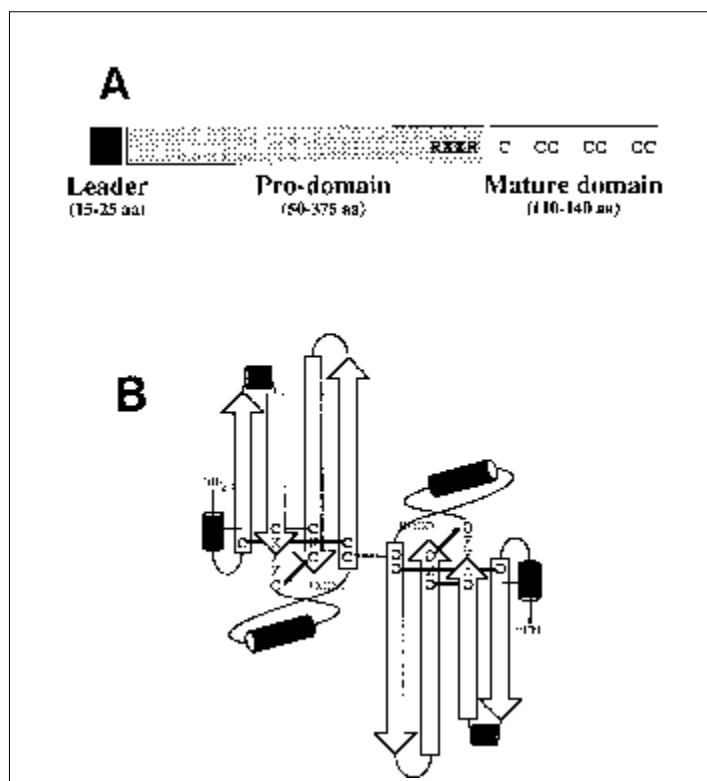
# **1 Introduction**

## **1.1 The TGF- $\beta$ superfamily**

The transforming growth factor (TGF- $\beta$ ) superfamily is a large family of highly conserved growth-regulatory polypeptides that includes the TGFs, activins, inhibins, bone morphogenetic proteins (BMPs), Müllerian-inhibiting substance, nodal, glial-derived neurotrophic factor, and lefty (Massagué 1990, 1997; Meno et al. 1996; Lin et al. 1993; Zhou et al. 1993; Roberts and Sporn 1990). Members of the TGF- $\beta$  superfamily exert a wide range of biological effects on a large variety of cell types: they regulate cell growth, differentiation, immune surveillance, matrix production and apoptosis. Many of them have important functions during embryonic development in pattern formation and tissue specification; in the adult they are involved in processes such as tissue repair and modulation of the immune system (reviewed by Heldin 1997).

### **1.1.1 The structural characteristics of the TGF- $\beta$ superfamily members**

The TGF- $\beta$  superfamily members are synthesized as larger precursor molecules with a signal sequence of 15-25 amino acids, a pro-domain of 50-375 amino acids, and a C-terminal mature region of 110-140 amino acids that mostly contains 7-9 cysteine residues (Fig. 1.1.A; Hogan et al. 1994). The C-terminal mature region can be released by being cleaved at the RXXR site. The mature, biologically active forms of TGF- $\beta$  superfamily members are typically homo- or heterodimers of two cysteine-rich, 12-15 kDa subunits, linked by a single disulfide bond (reviewed by Hoodless and Wrana 1998) (Fig. 1.1.B).



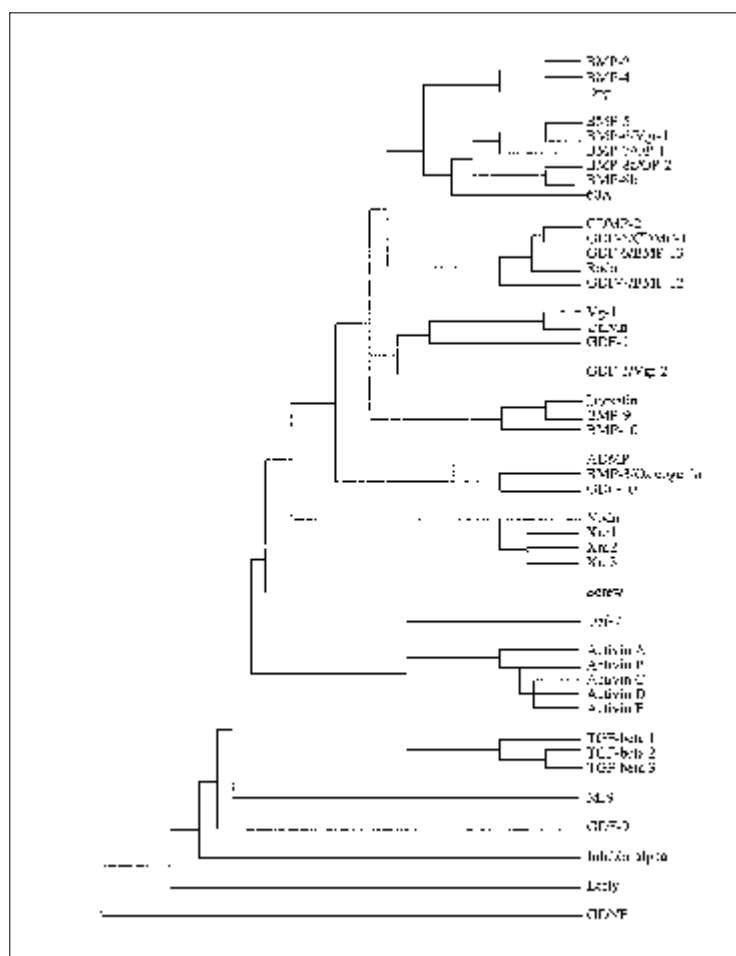
**Fig. 1.1:** Structure of TGF-β superfamily members.

(A) TGF-β-related proteins are synthesized as larger precursor proteins (Hogan et al. 1994; Kingsley 1994).

(B) Schematic structure of the mature region of TGF-β. Six of the characteristic cysteine residues form three disulfide bonds within each monomer subunit. The seventh cysteine forms a disulfide bond linking two monomers into a dimer (Kingsley 1994).

### 1.1.2 The members and subfamilies within the TGF-β superfamily

At present there are 43 proteins isolated from various species ranging from *Drosophila* to mammals that belong to the TGF-β superfamily. Based on comparison of the C-terminal mature regions the members can be classified into distinct subgroups with highly related sequences such as the TGFβ-subfamily, the activin-subfamily, the dpp-subfamily, the 60A-subfamily and the GDF-subfamily (Derynck and Feng 1997; Wozney and Rosen 1998).



**Fig. 1.2:** The TGF- $\beta$  superfamily. Most TGF- $\beta$ -related factors listed are of vertebrate origin except that DPP, 60A, and Screw are *Drosophila* proteins, univin is from sea urchin and Vg-1 is a *Xenopus* protein (Derynck and Feng 1997).

The Dpp and 60A subfamilies are related more closely to each other than to TGF- $\beta$ s or activins and have often been grouped together as part of a larger collection of molecules called DVR (dpp and Vg1 related) (Lyons et al. 1989) or BMP superfamily (Hogan 1996a). The *Drosophila decapentaplegic* (dpp) gene (Padgett et al. 1987), the BMP2 and BMP4 (Wozney et al. 1988; Wozney 1989), *Drosophila* gene 60A (Wharton et al. 1991; Doctor et al. 1992), BMP5, BMP6 or Vgr-1, BMP7 or osteogenic protein 1 (OP-1), BMP-8a or osteogenic

protein 2 (OP-2), and BMP-8b (Celeste et al. 1990; Lyons et al. 1989; Ozkaynak et al. 1990; Ozkaynak et al. 1992; Zhao et al. 1996) are all assigned to this subfamily.

### **1.1.3 Bone morphogenetic proteins**

The BMPs (bone morphogenetic proteins) have variously been called osteogenins, osteogenic proteins, DVR factors and BMPs (reviewed by Kingsley 1994). The name BMP was first given to three proteins purified from a demineralized bovine bone preparation that induced ectopic cartilage and endochondral bone formation when implanted in experimental animals (Wozney et al. 1988).

Now it is clear that the name BMP is misleading because there is strong genetic and experimental evidence that these molecules regulate biological processes as diverse as cell proliferation, apoptosis, differentiation, cell-fate determination, and morphogenesis. Moreover, the vertebrate BMPs are involved in the development of nearly all organs and tissues, as well as in critical steps in the establishment of the basic embryonic body plan (Hogan 1996). Although many roles have been assigned to the bone morphogenetic proteins, one of the key functions of BMP-like proteins in vertebrates is to induce formation of cartilage, bone, and the supporting tissues of the skeleton.

The use of purified individual bone morphogenetic protein molecules produced in recombinant systems has clarified the activity of many bone morphogenetic proteins. The implantation of recombinant human BMP2 with a suitable carrier of murine ectopic intramuscula or subcutaneous sites results in chondrogenesis and osteogenesis (Wang et al. 1990). Other bone morphogenetic proteins qualitatively show similar effects. Bone morphogenetic protein 4, as expected given its high degree of sequence similarity to BMP2, also induces new bone formation in this system. The less closely related molecules, BMP6 and BMP7 seem to induce bone at similar levels as BMP2, though a third member of this subgroup BMP5, requires

higher amounts of protein to induce similar amounts of bone (Gitelman et al. 1994; Sampath et al. 1992; Wozney and Rosen 1993).

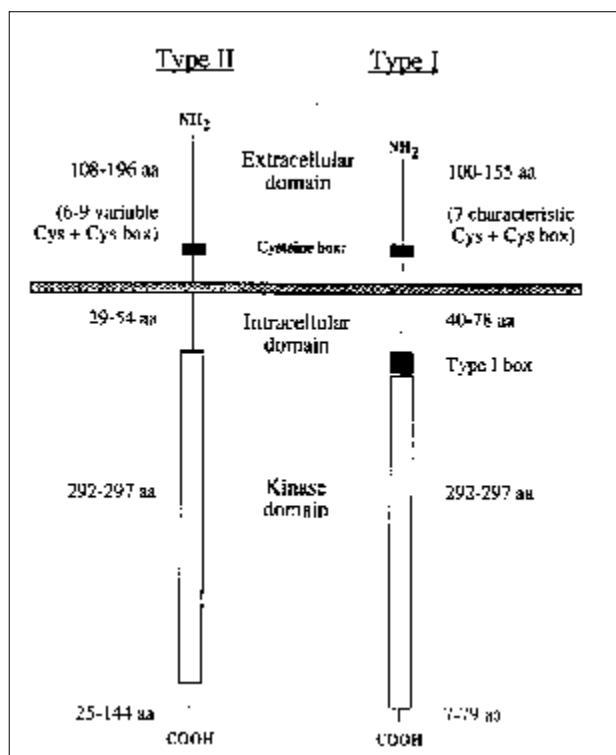
Many of the BMPs have been observed to affect differentiation of cells into cartilage and bone phenotypes by cell culture experiments examining the activities of bone morphogenetic proteins on various cell types, including embryonic and adult cells and cell lines derived from calvarial and long bone sources, in vitro (Ahrens et al. 1993; Rosen et al. 1996).

Identification of the regulatory elements controlling bone morphogenetic protein expression has shown that BMPs are integral parts of signaling cascades that involve several other gene families. Depending on the developmental system analysed, BMPs induce the expression of sonic and indian hedgehog, muscle segment homeobox genes, fibroblast growth factors and PTH/PTHrP receptors, genes shown to play fundamental roles in bone formation and chondrogenesis (Bitgood and McMahon 1995; Hattersley et al. 1995; Vortkamp et al. 1996; Zou and Niswander 1996). Conversely, sonic hedgehog, muscle segment homeobox genes, and retinoic acid all have been shown to affect BMP transcription and seem to be positive regulators of BMPs (reviewed by Hogan 1996a, b; Wozney and Rosen 1998).

### **1.2 The receptors of TGF- $\beta$ superfamily members**

Members of the TGF- $\beta$  superfamily signal through a conserved family of transmembrane serine/threonine kinase receptors, all of which have a short, cysteine-rich extracellular domain, which was called 'cysteine box' (Childs et al. 1993), a single hydrophobic transmembrane domain, and an intracellular serine/threonine kinase domain (Attisano and Wrana 1996; Massagué and Weis-Garcia 1996; Miyazono et al. 1994). Functional characterization and comparison of their primary amino acid sequences indicates that these receptors can be divided into two classes, the type I receptors and the type II receptors. Unique to type I receptors in the cytoplasmic domain is a highly conserved 30 amino acid region preceding the kinase

domain (Wrana et al. 1994b). This region contains a characteristic SGSGSG motif and is therefore known as 'GS domain', which is of crucial importance in signal transduction.



**Fig. 1.3:** Signaling receptors for TGF- $\beta$  superfamily members (reviewed by Kingsley 1994).

Considerable biochemical and genetic data have shown that signaling by TGF- $\beta$ -like factors occurs through heteromeric complexes of type I and type II receptors. In the absence of ligand, both type II and type I receptors appear to exist as independent homo-oligomers on the cell surface (ten Dijke et al. 1996). The type II receptor has constitutive kinase activity (Wrana et al. 1994a). Heterotetrameric complex formation is initiated when ligand binds to type II receptor, which then leads to recruitment of receptor I. This complex is likely to contain at least two molecules of each receptor (Weis-Garcia et al. 1996; Yamashita et al. 1994). The type II receptor then phosphorylates receptor I at serine and threonine residues within the 'GS domain' (Souchelnytskyi et al. 1996; Wieser et al. 1995). Phosphorylation of the GS domain

stimulates intracellular responses to the bound ligand (Wrana et al. 1994a; Attisano et al. 1996; Massagué and Weis-Garcia 1996).

### **1.3 The Smad family**

To identify signaling pathways that mediate the activities of TGF- $\beta$  family members, emphasis has been placed on the identification and characterization of proteins that associate with and are phosphorylated by the serine/threonine kinase receptors. Many proteins have therefore been identified including TRIP-1 (TGF- $\beta$ -receptor interacting protein-1), FT- $\alpha$  (farnesyl transferase- $\alpha$ ), apolipoprotein J/clusterin and FKBP12 (FK506/rapamycin binding protein) (Attisano and Wrana 1996) by yeast two-hybrid screens using the receptors as bait. These proteins may play a regulatory role in receptor function, but there is little evidence to indicate that they are actually required for signaling by TGF- $\beta$  ligands.

Genetic screens lead to two components which may be directly involved in TGF- $\beta$  family members signal transduction: a MAP kinase kinase kinase (TAK1, TGF- $\beta$ -activated kinase-1), and the family of proteins related to the *Drosophila* gene *Mothers against dpp* (MAD) (reviewed by Hoodless and Wrana 1998).

TAK-1 is a novel MAP kinase kinase kinase (MAPKKK). Its associated activator, TAB1 (TAK-1 binding protein), provides additional support for the induction of a kinase cascade by TGF- $\beta$  (Yamaguchi et al. 1995; Shibuya et al. 1996). TAK-1 has been shown to be activated by TGF- $\beta$  and BMP-4 but not EGF, and to induce transcriptional activation of the plasminogen activator inhibitor-1 promoter. Further investigation is necessary to clarify the role of the TAK-1 pathway in the signaling of TGF- $\beta$  superfamily.

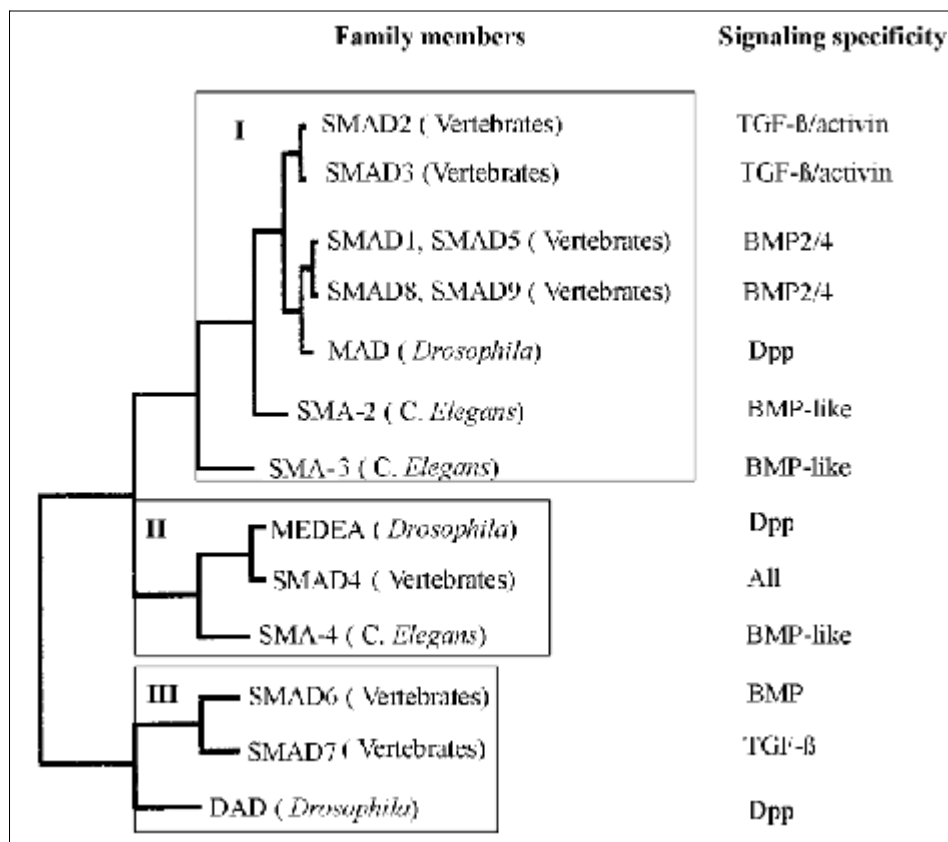
*Mothers against dpp* (MAD) and its homologs have been characterized recently as important signaling mediators of the responses to TGF- $\beta$ -related factors (Raftery et al. 1995). This led to



a breakthrough in understanding how signals are transduced from serine/threonine kinase receptors to the nucleus.

### 1.3.1 The members of Smad protein family

The first family member, the *Drosophila Mad*, was identified in a screen for maternal effect enhancers of mutations in *dpp* (Raftery et al. 1995). The related homologs of Mad have been identified in *C. elegans*, human, mouse, rat, and *Xenopus* (Savage et al. 1996; Derynck and Zhang 1996b; Massagué 1996; Wrana and Attisano 1996). In an attempt to simplify the nomenclature, the designation *smad*, an incorporation of *sma*- and *Mad*- gene names, has been suggested for vertebrate homologs of *sma* and *Mad* (Derynck et al. 1996a).



**Fig. 1.4:** Members and signaling specificity of the Smad family (Based on Padgett et al. 1998, Kretzschmar & Massagué 1998 and Massagué et al. 1997). I: receptor-regulated Smads; II: co-Smads; III: anti-Smads.

On the basis of structural and functional criteria, the Smad family can be divided into three subgroups (Fig. 1.4). The first group comprises those Smads that are direct substrates of the TGF- $\beta$  family receptor kinases, which are called receptor-regulated Smads (Padgett et al. 1998); The second group, co-Smads, includes Smads that are not direct receptor substrates but participate in signaling by associating with receptor-regulated Smads. Smad4, *sma-4* and *Drosophila Medea* belong to this group (Liu et al. 1997; Das et al. 1998; Hudson et al. 1998). The third group consists of Smad6, Smad7 and *Drosophila Daughters against dpp* (Dad), they inhibit Smad activation and therefore are referred to as anti-Smads (Kretzschmar and Massagué 1998; Inoue et al. 1998).

### 1.3.2 Smad protein domains and their function

Smad proteins are molecules of relative molecular mass 42K-60K. They are highly conserved across species, but have no known structural motifs and bear no resemblance to components of other signaling pathways (Heldin et al. 1997). All members of this family typically consist of three regions, each of them encompassing roughly one third of the molecular structure.

The highest level of amino acid sequence similarity is found in the N-terminal and C-terminal regions, which are referred to as the N and C domains (or the MH1 and MH2 domains), respectively. In the C domain of the receptor-regulated Smads there is a conserved sequence SSXS, which can be phosphorylated by type I receptor kinase (Macías-Silva et al. 1996; Kretzschmar et al. 1997a). The C domain is regarded as an effector domain (Meersseman et al. 1997).

In the basal state, the presence of the N domain inhibits the transcriptional and biological activities of Smad C domains (Hata et al. 1997). But the N domain may not have a purely inhibitory function. The N domain of *Drosophila Mad* has specific DNA-binding activity

which is required for DPP-induced activation of an enhancer within the *vestigial* wing-patterning gene (Kim et al. 1997).

The central proline-rich region (referred to as the linker region or P domain) is more divergent and of variable length. However, Smad linker regions are highly conserved between close isoforms. Four repeated PXSP motifs in the linker region of Smad1 can be phosphorylated by the Erk family of mitogen-activated protein kinases which then prevent nuclear accumulation of Smad1 and the transcriptional activity of Smad1 in response to BMP (Kretzschmar et al. 1997b).

### **1.3.3 Functional specificity of Smads**

Studies on Smads have defined the specificity of these proteins as mediators of TGF- $\beta$ -family signals. Different Smads may mediate different ligand signals: Smad1 and its close homologs Smad5 and Smad8 are mediators of BMP signals (Yingling et al. 1996; Liu et al. 1996; Lechleider et al. 1996; Kretzschmar et al. 1997b; Hoodless et al. 1996; Graff et al. 1996). The recently described Smad9/MADH6 is structurally similar to Smad1 and may also be involved in BMP signaling (Watanabe et al. 1997). Smad2 and Smad3 are substrates and mediators of related TGF- $\beta$  and activin receptors in vertebrates (Baker and Harland 1996; Eppert et al. 1996; Graff et al. 1996; Macías-Silva et al. 1996). Smad4 mimics the BMP-like effects as well as the activin/Vg1-like effects (Lagna et al. 1996). When overexpressed in mammalian cells, Smad6 shows a very specific inhibition of BMP but not activin/TGF- $\beta$  signaling (Hata et al. 1998; Imamura et al. 1997). Transfection of Smad7 blocks activin/TGF- $\beta$  signaling (Nakao et al. 1997; Hayashi et al. 1997).

### **1.3.4 Activation of Smads**

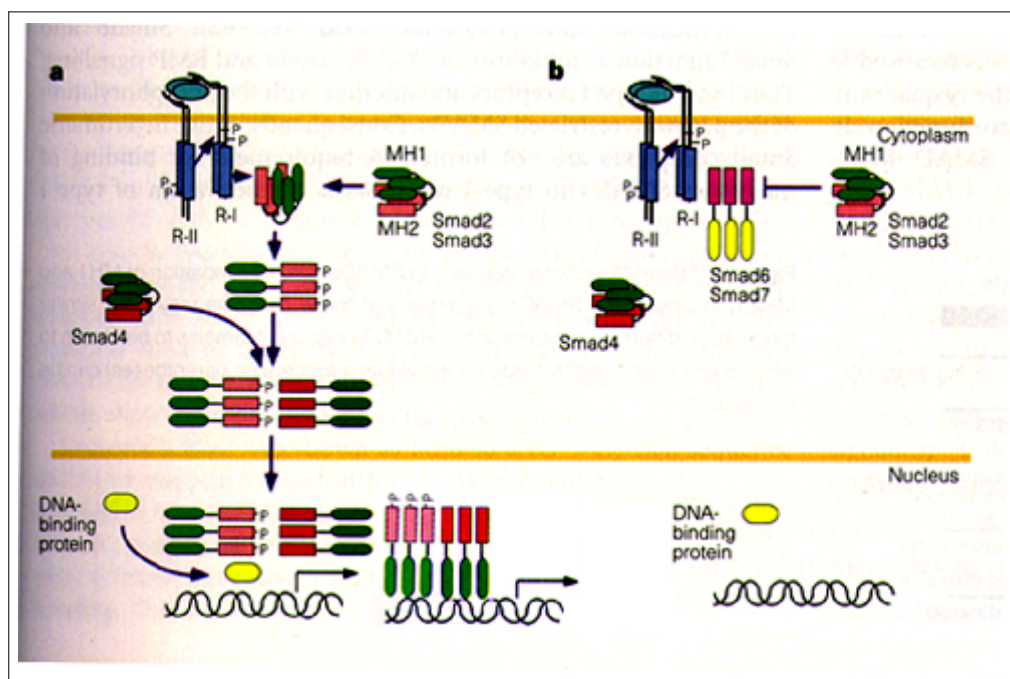
Maximum transcriptional effect requires the cooperation between receptor-regulated Smads and Smad4 (Lagna et al. 1996; Zhang et al. 1996). Activation of type I receptors triggers the assembly of heteromeric complexes of these two types of Smads by phosphorylation of receptor-regulated Smads in their C-terminal SSXS motifs. The mechanism may involve a phosphorylation-induced unfolding of the N- and C-terminal domains, allowing interaction with Smad4 to occur (Souchelnytskyi et al. 1997). Given the trimeric structure of Smad4 (Shi et al. 1997), such complexes are hexamers, whose composition depends on the pathway that has been activated.

### **1.3.5 Smads function in the nucleus**

Smads are present in both the cytoplasm and nucleus (Hoodless et al. 1996; Newfeld et al. 1996; Graff et al. 1996; Liu et al. 1996). Ligand binding increases accumulation of Smads in the nucleus (Baker and Harland 1996). Smad4 has an important function in mediating this process (Zhang et al. 1997). The translocation seems to require phosphorylation of the receptor-regulated Smads and that N domain may play an inhibitory function by maintaining them in the cytoplasm, possibly by association with cytosolic components (Baker and Harland 1996). By now two possibilities of nuclear functions of Smads have been referenced: (i) Smads as coactivators. Two transcription factors have been implicated in downstream signaling of the Smads. One is FAST-1 (forkhead activin signal transducer-1). Smad2 can complex with FAST-1 and then transcriptionally activate the promoter of the gene *Mix-2* when co-injected with activin mRNA (Chen, Y. et al. 1996). The other is a zinc-finger transcription factor encoded by the *Drosophila* gene *schnurri* (*shn*) (Arora et al. 1995; Grieder et al 1995). *Shn* activity appears to be regulated by Dpp. (ii) Smads bind DNA directly. The N domain of *Drosophila* Mad can itself bind to elements within the *vestigial* (*Vg*) promoter, suggesting a sequence-specific binding function that might directly account for the biologic activity of the Mad protein (Kim et al. 1997). Smad3 and Smad4 have also been shown to bind DNA in a sequence-specific manner (Yingling et al. 1997; Zawel et al. 1998).

### 1.3.6 A model of Smad function in TGF- $\beta$ signaling

Taken together, the current findings invite a model of receptor signaling and Smads function (Fig. 1.5), much of which still requires experimental evaluation (Derynck and Zhang 1996; Derynck and Feng 1997; Heldin et al. 1997; Hu et al. 1998; Kretzschmar and Massagué 1998).

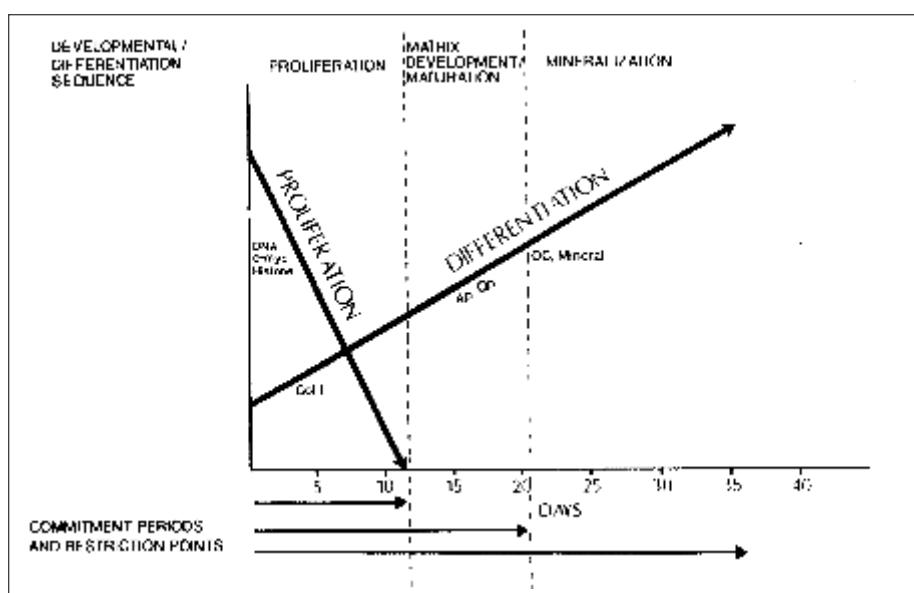


**Fig. 1.5:** Proposed model for the Smad-dependent TGF- $\beta$  signal transduction pathway (Based on Heldin et al. 1997; Hu et al. 1998).

## **1.4 The *in vitro* model of osteoblast-differentiation**

There are three major cell types contributing to the skeleton: chondrocytes, which form cartilage; osteoblasts, which deposit bone matrix and once embedded in bone matrix they would mature into terminally differentiated osteocytes; and osteoclasts, which resorb bone. Chondrocytes and osteoblasts are of mesenchymal origin, whereas osteoclasts derive from the hematopoietic system (Erlebacher et al. 1995).

Many extracellular factors were identified to be able to modulate mesenchymal differentiation, including PTH (parathyroid hormone), GDF, and collagens. Embryonic primary cells or pluripotent mesenchymal cells are used in *in vitro* studies, which aim at identifying transcription factors or clarifying the early events in the differentiation of osteoblasts and chondrocytes. With a cell culture of primary osteoblasts, Owen and Stein have shown the relationship between proliferation and differentiation during the rat osteoblast developmental sequence and developed a model (Owen et al. 1990). Accordingly the process comprises three distinct time periods. (i) The proliferative phase, lasts for the first 10-12 days and is characterised by the expression of genes associated with cell division e.g. H4 histone, and cell growth, e.g. *c-fos* and *c-myc*. A number of genes associated with the development of connective tissue, i.e. type I collagen, TGF- $\beta$  and fibronectin are also expressed in this phase. (ii) The matrix development and maturation phase, takes place over days 12-21 and is characterised by an increase in the expression of alkaline phosphatase, osteopontin and matrix Gla protein (MGP). (iii) The mineralization phase, is characterized by the progressive expression of two bone-related genes, osteopontin and osteocalcin, whose expression is followed by matrix mineralization.



**Fig. 1.6:** The relationship between proliferation and differentiation during the rat osteoblast developmental sequence. The three principal periods of the osteoblast developmental sequence are designated within broken vertical lines. Commitment periods and restriction points indicated were experimentally established (Owen et al. 1990).

There are two restriction points in the osteoblast developmental sequence at which important regulatory signals must be received before the process can proceed further. The first restriction point occurs when the proliferation is down-regulated and matrix maturation is induced and the second when mineralization starts. The mechanism how the important signals regulate the restriction points is unclarified, but BMPs might represent potential candidates in the regulation.

## 1.5 Research aims

BMP2 has been shown to induce cartilage and bone in both ectopic and orthotopic sites *in vivo* and to have many different effects on multiple cell types *in vitro* (Hiraki et al. 1991; Chen et al. 1991; Takuwa et al. 1991; Yamaguchi et al. 1991). The murine fibroblastic C3H10T1/2 cells, which have been established from an early stage mouse embryo (Reznikoff et al. 1973), have been shown to be able to differentiate into various mesenchymal pathways giving rise to myoblasts, adipocytes, chondrocytes and osteoblasts (Taylor and Jones 1979; Wang et al. 1994; Ahrens et al. 1993). Their responses towards TGF- $\beta$  and BMP-treatment make this cell line a useful model system to explore the involvement of factors in various mesenchymal differentiation processes.

Signaling of TGF- $\beta$  and BMPs from membrane receptors into the nucleus is mediated by the recently identified Smad protein family. The BMP signal transducer Smad1 exhibits a domain-like organization. The C domains of receptor-regulated Smads harbour the effector domain; N domains can bind to DNA directly in addition to their inhibitory functions; the role of the proline-rich linker region is unclear. The C3H10T1/2 developmental *in vitro* model system could contribute to elucidating the functions of the three domains, especially the function of the linker region of Smad1 in the mesenchymal differentiation processes, particularly with regard to the osteo/chondrogenic development. For this purpose the C3H10T1/2 cells should recombinantly express Smad1 or Smad1 domains. The resulting effects on osteo/chondrogenic development should be analyzed by histochemical and genetic analyses of marker genes typical or specific for osteogenesis, chondrogenesis, but also adipogenesis.

Smads are transcriptional activators, and their phosphorylation and translocation to the nucleus are thought to affect transcriptional regulation of BMP-responsive genes. The exact mechanism by which Smads regulate transcriptional in-/activation of marker genes is an important question whose answer will further define the role of Smads in BMP signaling. In



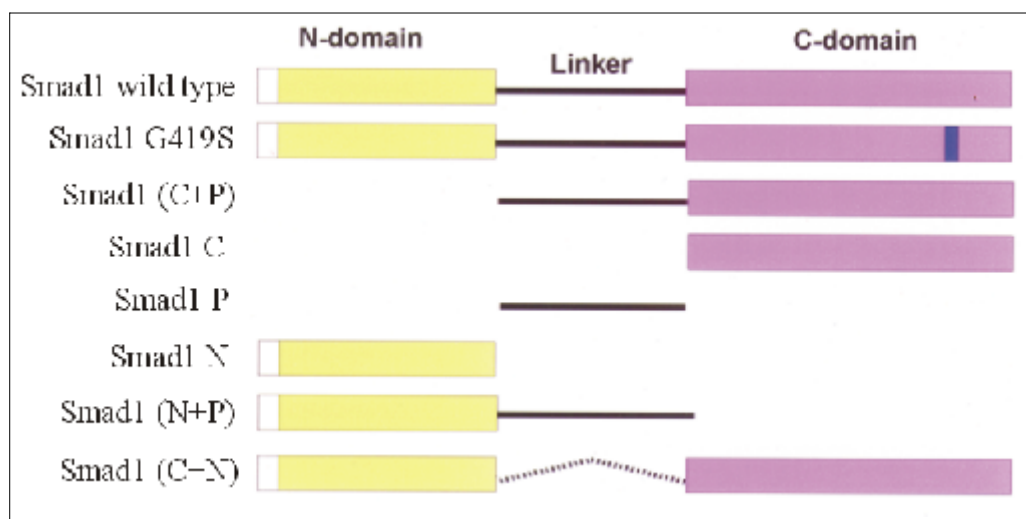
this regard, the reporter gene assays under the control of promoters from genes like Msx-1, 3TP-Lux, PTH/PTHrP receptor and JunB should be characterized.

## 2 Results

### 2.1 Stable recombinant expressions of Smad1 or Smad1-domains into murine mesenchymal progenitor C3H10T1/2 and BMP2 expressing C3H10T1/2 cells

#### 2.1.1 Smad1 and its domains

The *Xenopus* Smad1 is a polypeptide of 466 amino acids with two conserved domains: the N-terminal domain and the C-terminal domain, also called MH1 and MH2 (Hoodless et al. 1996), separated by a proline-rich variable domain: the linker region (reviewed by Kretzschmar and Massagué 1998).



**Fig. 2. 1:** Smad1 and its domains.

The *Xenopus* Smad1 wild type and deletion mutants were established and obtained from D. Huylebroeck Laboratory of Molecular Biology, Celgen, University of Leuven. To analyze the functions of Smad1's three domains, we have looked at the following domain mutants: Smad1 lacking the N terminal amino acids 1-145 (Smad1 (C+P) domain); Smad1 without amino acids

1-262 (Smad1 C domain); Smad1 deleted in amino acids 263-466 (Smad1 (N+P) domain); Smad1 lacking amino acid 145-466 (Smad1 N domain); the fragment containing amino acids 146-262 (Smad1 linker region or Smad1 P domain); and Smad1 lacking amino acids 146-262 (Smad1 (C+N) domain) (Fig. 2.1). In addition, the point mutant G419S, which interferes with BMP2-dependent Smad1 phosphorylation, was also tested in our experiments.

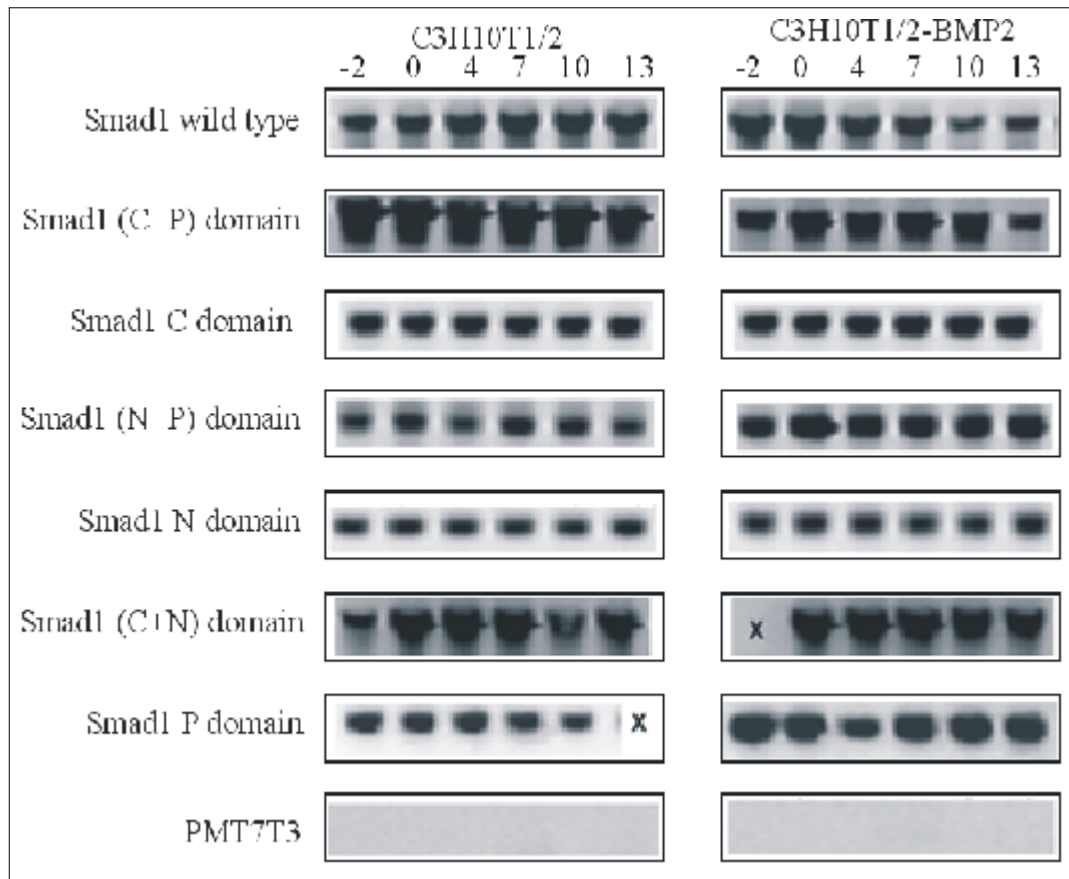
### **2.1.2 Recombinant expression of full-length Smad1 and Smad1 domains in C3H10T1/2 and BMP2 expressing C3H10T1/2 cells**

The Smad1 constructs were permanently transfected into the murine mesenchymal precursor C3H10T1/2 cells and the BMP2-transfected C3H10T1/2 cells (C3H10T1/2<sub>BMP2</sub>) according to protocol 5.5.6.1 and thereby were stably integrated into the genomes of these cell lines. The empty expression vector pMT7T3 was transfected into these cells to establish control cell lines.

The cells (in 12-well-plates) were transfected with 1.5 µg of the expression vectors described above, and 150ng selection plasmid pAG60 (*neo*<sup>R</sup>-selection plasmid, Colbe're-Garapin et al. 1981) using DOSPER (Boehringer) (Chapter 5.5.6.1). The integration of the selection plasmid into genomes enables the transfected cells to survive in selection medium containing G418 (500 µg/ml). The control transfection was performed with empty expression vectors. Cells started to die 7 days after G418 was added to the medium. Within two weeks of selection, usually 4-10 G418-resistant clones were observed. Single clones were picked and subcultivated. The selection pressure was maintained during the entire cultivation period.

To check the extent and stability of the expression of various Smad1 constructs in the transfected C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells, the various Smad1 constructs and the control plasmid transfected cells were plated in Roux bottles (80 cm<sup>2</sup>). After reaching confluence (arbitrarily termed day 0), differentiation medium (DMEM with 50 µg/ml ascorbic acid and 10 mM β-glycerolphosphate) was used for further cultivation. At the indicated time intervals (2 days before confluence and 0, 4, 7, 10, 13 days postconfluence) the cells were

harvested and total RNA was isolated by Tri-Reagent<sup>®</sup>LS (Chapter 5.5.7). SuperScript<sup>™</sup> Reverse transcriptase was used to synthesize cDNA (Chapter 5.4.1). RT-PCR (Chapter 5.4.2) were performed with different primers, which are specific for *Xenopus* Smad1 domains, to verify the expression of recombinant Smad1 constructs in C3H10T1/2 cells and C3H10T1/2<sub>BMP2</sub> cells.



**Fig. 2.2:** RT-PCR analyses of recombinant Smad1 and its domains in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells. Cultivation was 13 days, X = RNA pellet was lost during isolation.

Fig. 2.2 shows the recombinant stable expression of *Xenopus* Smad1 and Smad1 domains in murine C3H10T1/2 cells and in C3H10T1/2<sub>BMP2</sub> cells. In the control cells Smad1 is present in an endogenous way (Fig. 2.34) but was not detected with the primers for *Xenopus* Smad1. The level of expression was comparable.

The inactive human Smad1 point mutant G419S was also transfected into C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells to test whether the conserved 419 glycine of Smad1 has a function in the signal transduction. Fig. 2.3 shows the recombinant stable expression of Smad1<sup>G419S</sup> in murine C3H10T1/2 and in C3H10T1/2<sub>BMP2</sub> cells. With the human-specific Smad1 primer no signal was detected in the control cells, although Smad1 is endogenously expressed in the control cells.

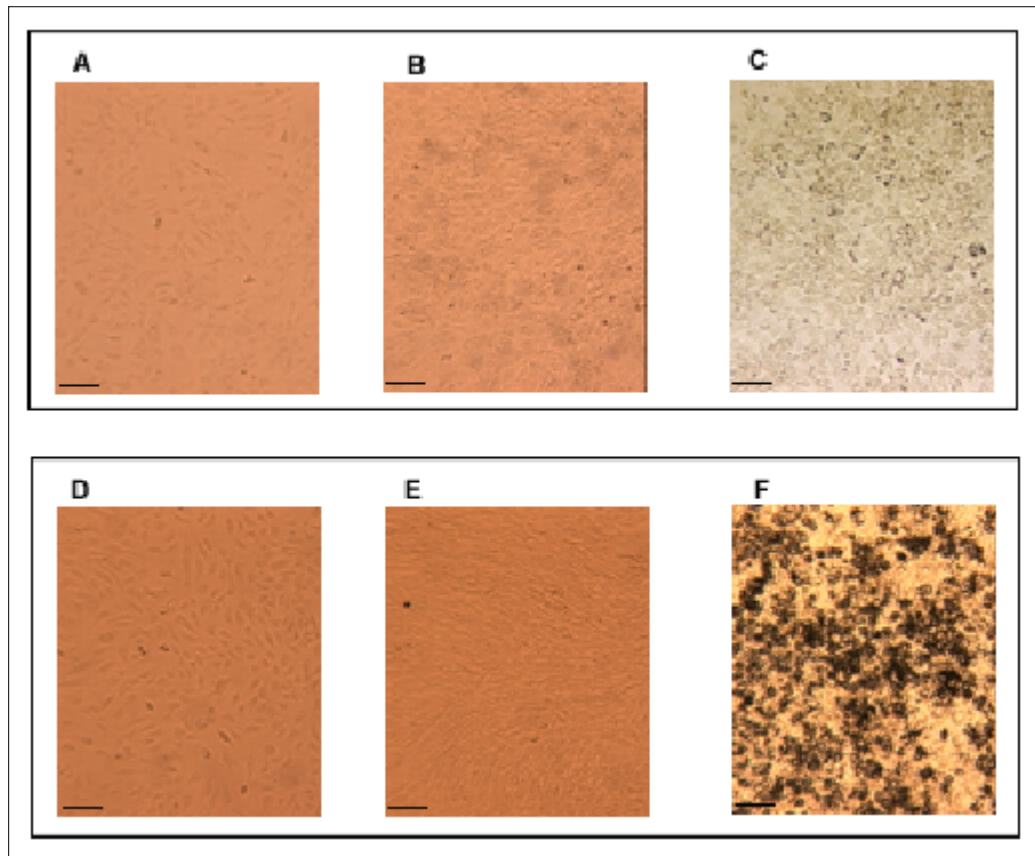


**Fig. 2.3:** RT-PCR analyses of recombinant Smad1<sup>G419S</sup> in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells. Cultivation was 13 days.

## 2.2 The morphological changes of cells expressing recombinant full-length Smad1 and Smad1 domains

C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells recombinantly expressing control vector, Smad1 wild type, Smad1 (C+P) domain, Smad1 C domain, Smad1 (N+P) domain, Smad1 N domain, Smad1 (N+C) domain, and Smad1 P domain were grown in 10% FCS DME-medium containing the appropriate selection antibiotics (G418 and/or puromycin). The cells were plated at a density of 7500 cells /cm<sup>2</sup>. After reaching confluence 50 µg/ml ascorbic acid and 10 mM β-glycerophosphate were added as specified in the protocol of Owen (1990) for the cultivation of native osteoblast-like cells. Ascorbic acid supports the synthesis of collagen (Jeffery and Martin 1966), and β-glycerolphosphate serves as an additional source of organic phosphate enabling mineralization (Owen et al. 1990). The morphological differences among

the control cells and cells expressing recombinant Smad1 and Smad1 domains were documented by light microscopy.

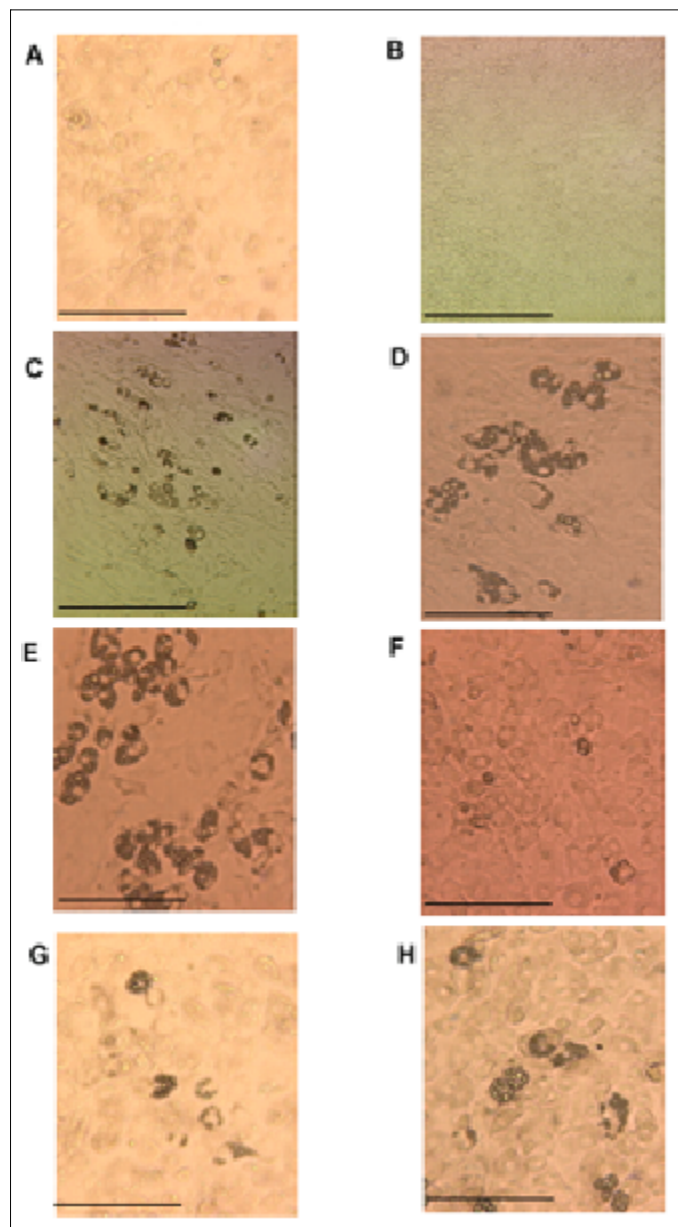


**Fig. 2.4:** The cell morphology of control cell lines: C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells

- A** C3H10T1/2 cells, 2 day before confluence
- B** C3H10T1/2 cells, at confluence
- C** C3H10T1/2 cells, 13 days post-confluence
- D** C3H10T1/2<sub>BMP2</sub> cells, 2 day before confluence
- E** C3H10T1/2<sub>BMP2</sub> cells, at confluence
- F** C3H10T1/2<sub>BMP2</sub> cells, 13 days post-confluence
- 100  $\mu$ m.

The C3H10T1/2 control cells grew in monolayer, they showed a typical fibroblastic morphology during proliferation (Fig. 2.4 A); Upon reaching confluence the cells changed their morphologies in differentiation medium. The cells were closely packed together (Fig. 2.4

B). 5 or 6 days post-confluence the cell morphology changed from fibroblastic to cuboidal-shaped cells (Fig. 2.4 C). The C3H10T1/2<sub>BMP2</sub> cells lost their original fibroblastic nature upon reaching confluence, grew in multilayers (Fig 2.4 E). In addition, many adipocytes (fat cells) were observed in the C3H10T1/2<sub>BMP2</sub> cells starting from 7-9 days post-confluence. The adipocytes were identified by their characteristic phenotypes: there are many lipid droplets in the cytoplasm (Fig. 2.4 F).



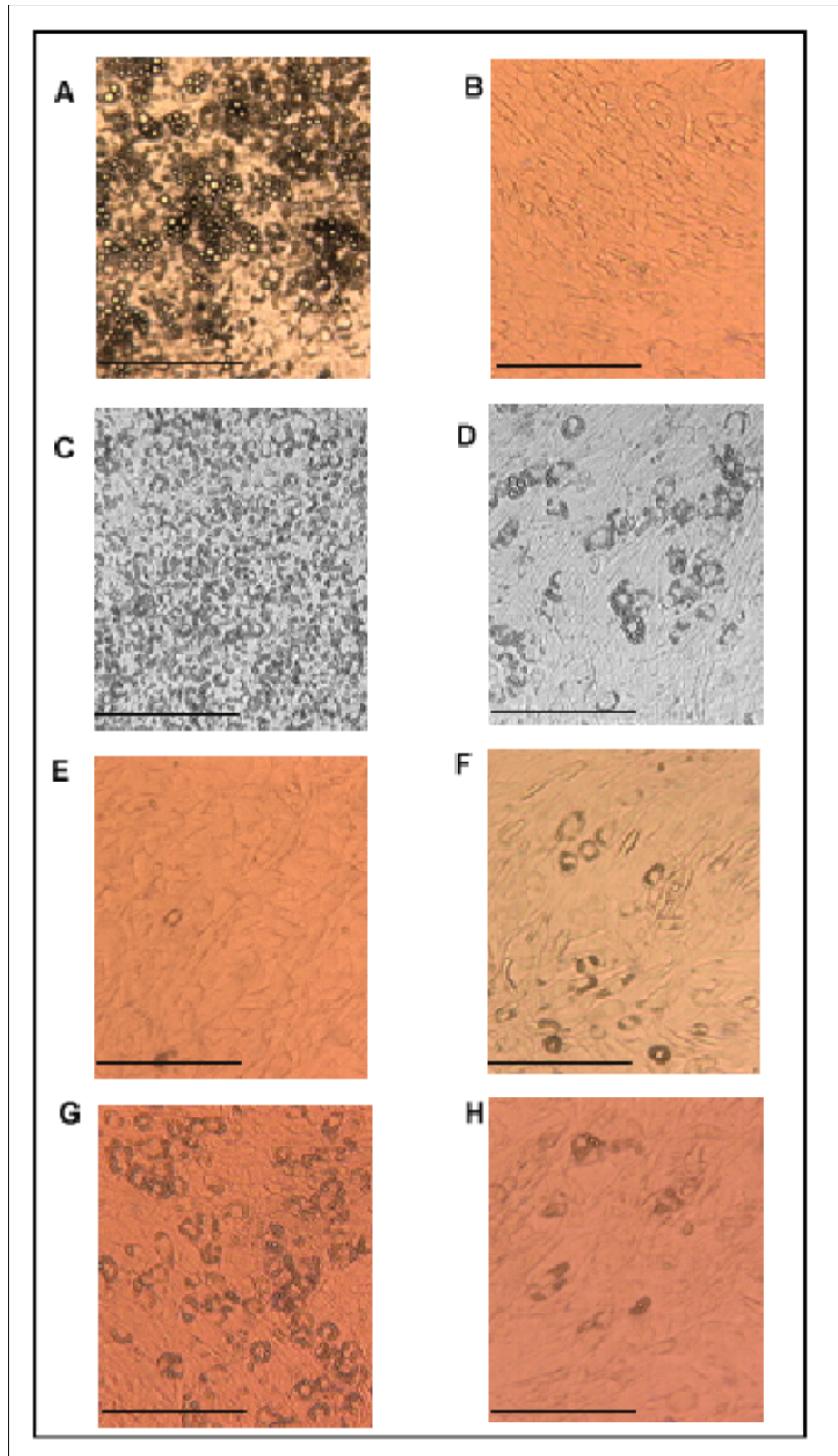
**Fig. 2.5:** The cell morphology of C3H10T1/2 cells recombinantly expressing Smad1 and Smad1 domains (10 days postconfluence). — 100  $\mu$ m.

- A: Parental C3H10T1/2
- B: C3H10T1/2 - Smad1 wt
- C: C3H10T1/2 - Smad1(C+P)
- D: C3H10T1/2 - Smad1 C
- E: C3H10T1/2 - Smad1(N+P)
- F: C3H10T1/2 - Smad1 N
- G: C3H10T1/2 - Smad1(C+N)
- H: C3H10T1/2 - Smad1 P

C3H10T1/2 cells expressing recombinant wild type Smad1 (C3H10T1/2-Smad1 wt) grow in monolayer and have a fibroblastic appearance. Adipocytes were not detected in this cell line. However, adipocyte formation was observed in C3H10T1/2 cells expressing Smad1 domains (Fig. 2.5 C-H)

During proliferation, all C3H10T1/2<sub>BMP2</sub> cells expressing recombinant Smad1 domains have a comparable appearance. Upon reaching confluence, these cell lines grew in multilayers and lost their original fibroblastic phenotypes. Morphological differences among various cell lines were first observed at 7 days post-confluence: The C3H10T1/2<sub>BMP2</sub> cells expressing Smad1 wild type (C3H10T1/2<sub>BMP2</sub>-Smad1 wt), C domain (C3H10T1/2<sub>BMP2</sub>-Smad1 C), (N+P) domain (C3H10T1/2<sub>BMP2</sub>-Smad1 (N+P)), N domain (C3H10T1/2<sub>BMP2</sub>-Smad1 N), (C+N) domain (C3H10T1/2<sub>BMP2</sub>-Smad1 (C+N)) and P domain (C3H10T1/2<sub>BMP2</sub>-Smad1P) showed reduced amounts of adipocytes (Fig. 2.6 B, D, E, F, G, H); in contrast, expression of the Smad1 (C+P) domain in C3H10T1/2<sub>BMP2</sub> cells (C3H10T1/2<sub>BMP2</sub>-Smad1 (C+P)) resulted in a thick layer of adipocytes (Fig. 2.6 C, A).





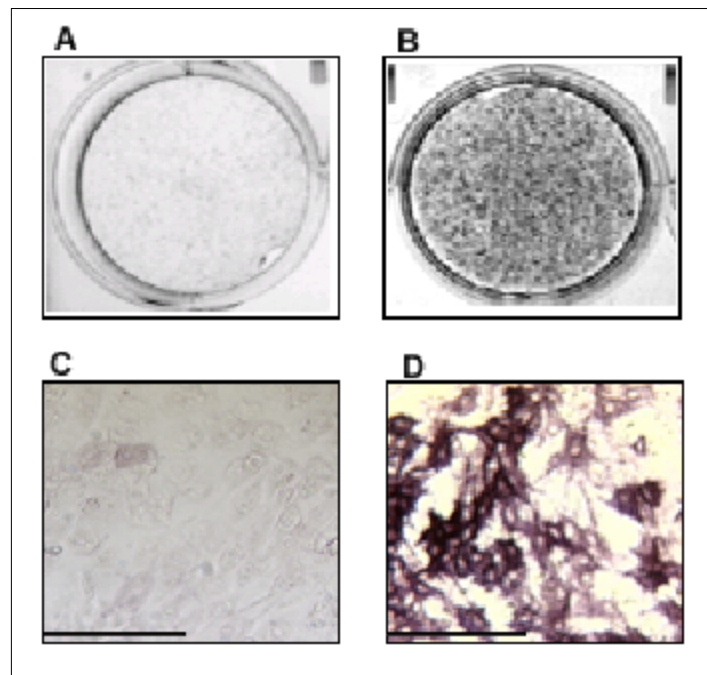
**Fig. 2.6:** The cell morphology of C3H10T1/2<sub>BMP2</sub> cells expressing Smad1 or its domains (10 days postconfluence). — 100  $\mu$ m.

- A: control C3H10T1/2<sub>BMP2</sub>
- B: C3H10T1/2<sub>BMP2</sub> - Smad1 wt
- C: C3H10T1/2<sub>BMP2</sub> - Smad1(C+P)
- D: C3H10T1/2<sub>BMP2</sub> - Smad1 C
- E: C3H10T1/2<sub>BMP2</sub> - Smad1(N+P)
- F: C3H10T1/2<sub>BMP2</sub> - Smad1 N
- G: C3H10T1/2<sub>BMP2</sub> - Smad1(C+N)
- H: C3H10T1/2<sub>BMP2</sub> - Smad1 P

### 2.2.1 Smad1-domain-dependent formation of osteoblast-like cells identified by alkaline phosphatase staining

The two control cell lines (C3H10T1/2 and C3H10T1/2<sub>BMP2</sub>) and the cells expressing recombinant full-length Smad1 and Smad1 domains were plated at a density of 7500 cells/cm<sup>2</sup> in 6-well-plates. Cells were grown in DMEM supplemented with 10% FCS and G418 or/and puromycin. After reaching confluence, differentiation medium was used for further cultivation. At the indicated time intervals the cells were fixed (Chapter 5.5.5) and alkaline phosphatase staining were performed with Sigma Fast BCIP/NBT tablets (Chapter 5.5.9.1).

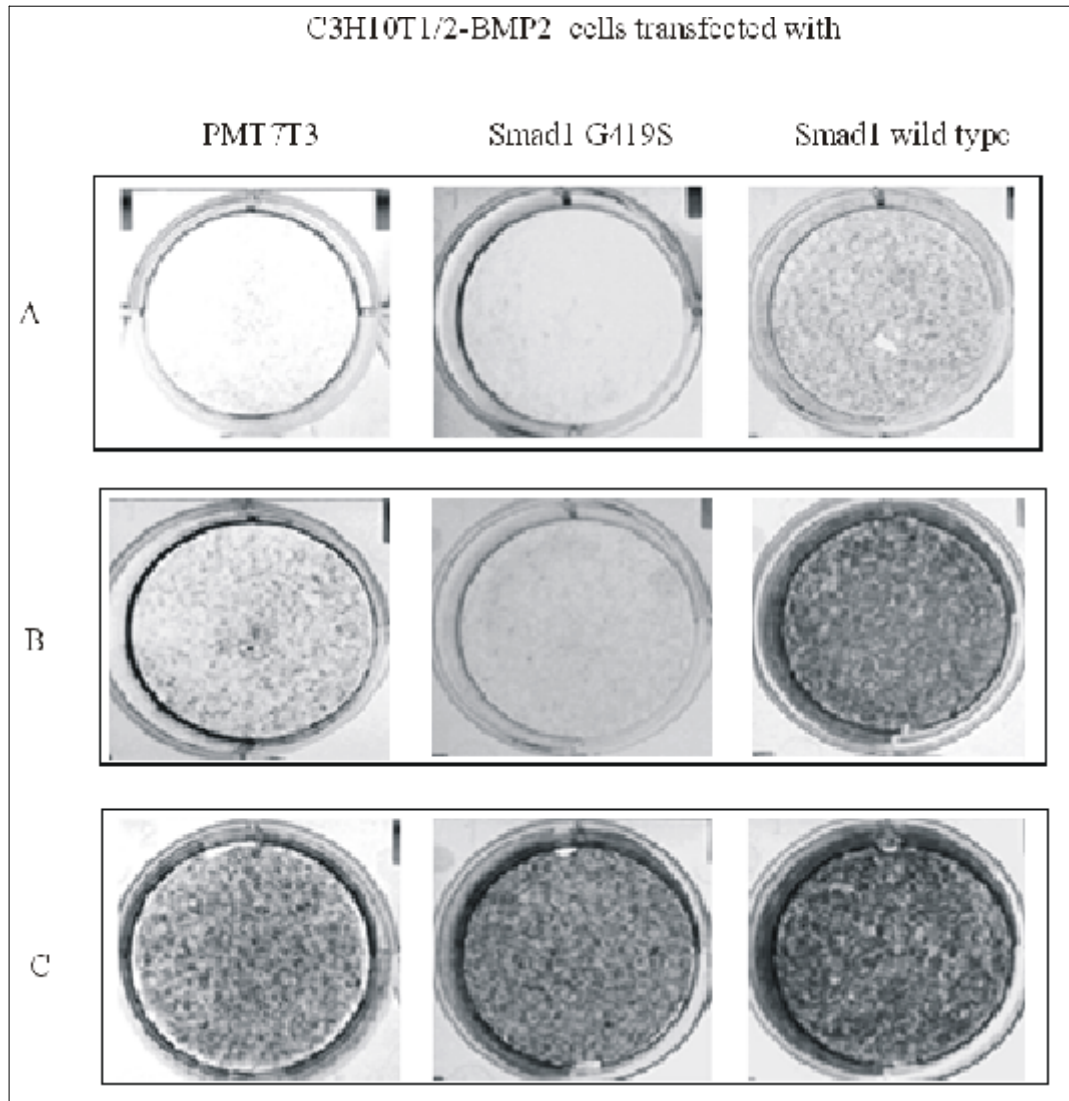
Alkaline phosphatase positive cells exhibiting a stellate ‘osteoblastic’ phenotype could first be identified at 4 days post-confluence in C3H10T1/2<sub>BMP2</sub> cells. The number of positive cells increased until the end of the cultivation (Fig. 2.7 B, D). In contrast, the parental C3H10T1/2 cells showed only a few faintly stained cells (Fig. 2.7 A, C).



**Fig. 2.7:** Osteoblast-like cells identified by alkaline phosphatase staining of the C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells (13 days post-confluence). — 100  $\mu$ m.

- A:** C3H10T1/2
- B:** C3H10T1/2<sub>BMP2</sub>
- C:** C3H10T1/2 cells, 275-fold magnification
- D:** C3H10T1/2<sub>BMP2</sub> cells, 275-fold magnification

Fig. 2.8 showed that consistent with the idea that Smad1 is a signaling mediator for BMP2, the number of alkaline phosphatase positive cells was higher in C3H10T1/2<sub>BMP2</sub>-Smad1 wt cells than in C3H10T1/2<sub>BMP2</sub> control cells. This stimulatory effect was abolished in C3H10T1/2<sub>BMP2</sub>-Smad1<sup>G419S</sup> cells (Fig. 2.8), confirming that wild type Smad1 has the ability to stimulate BMP2-dependent osteogenic differentiation in C3H10T1/2 cells, while the G419S mutation in Smad1 suppresses differentiation, but does not exert a dominant-negative effect.



**Fig. 2.8:** Osteoblast-like cells identified by alkaline phosphatase staining of C3H10T1/2<sub>BMP2</sub> cells, C3H10T1/2<sub>BMP2</sub>-Smad1<sup>G419S</sup> cells, and C3H10T1/2<sub>BMP2</sub>-Smad1 wt cells.

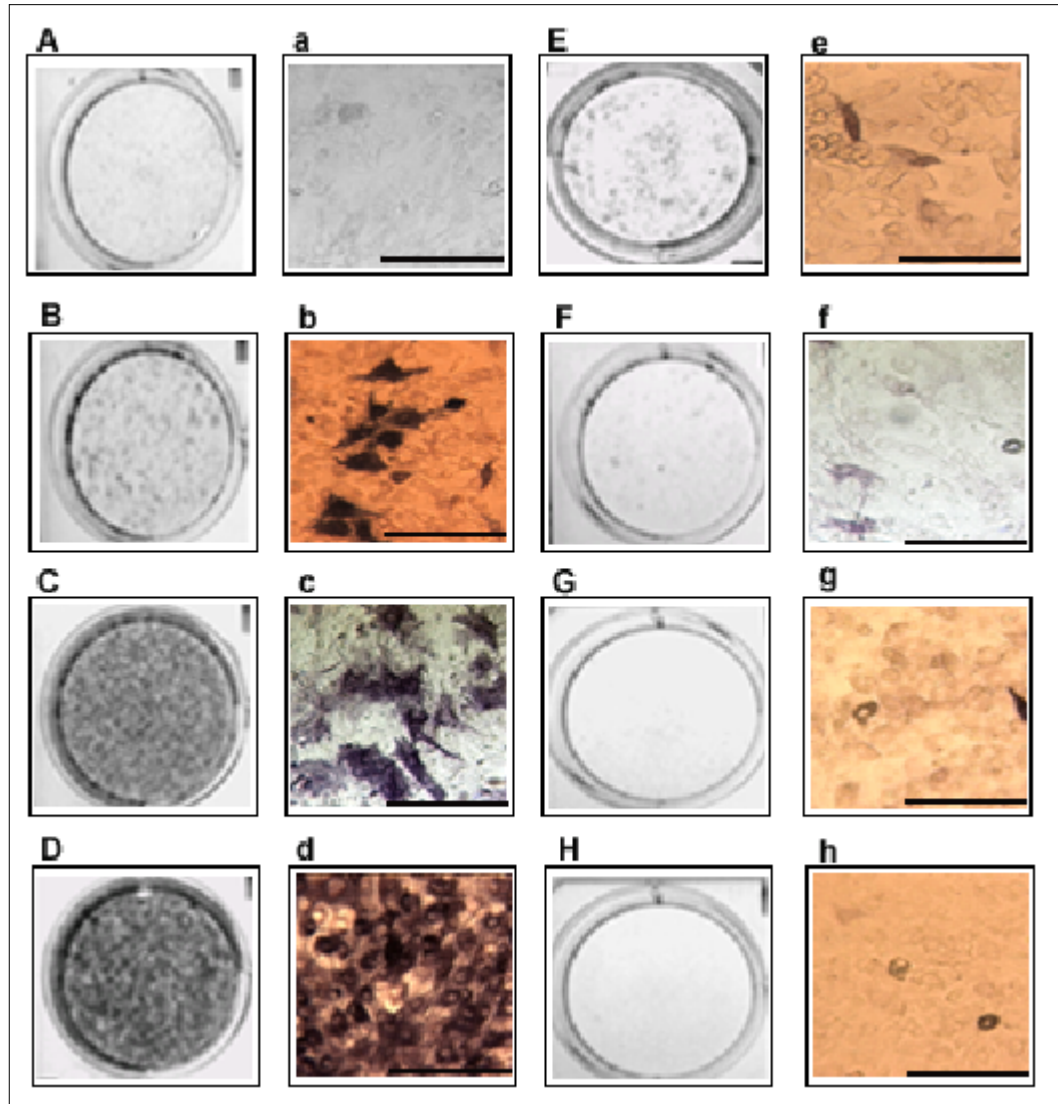
**A:** day 0

**B:** 4 day post-confluence

**C:** 13 day post-confluence

The recombinant expression of the Smad1 (N+P) domain, N domain, (C+N) domain and P domain did not lead to the development of cells exhibiting alkaline phosphatase activity in parental C3H10T1/2 cells (Fig. 2.9 A, a; E, e; F, f; G, g; H, h). Only a few stained cells were observed in C3H10T1/2-Smad1 wt cells (Fig. 2.9 B, b) whereas the C3H10T1/2-Smad1 (C+P)

cells and C3H10T1/2-Smad1 C cells showed high numbers of alkaline phosphatase-positive cells (Fig. 2.9 C, c; D, d).



**Fig. 2.9:** Osteoblast-like cells identified by alkaline phosphatase staining of C3H10T1/2 cells expressing recombinant Smad1 or Smad1 domains (10 day post-confluence).

— 100  $\mu$ m.

**A, a:** C3H10T1/2 cells;

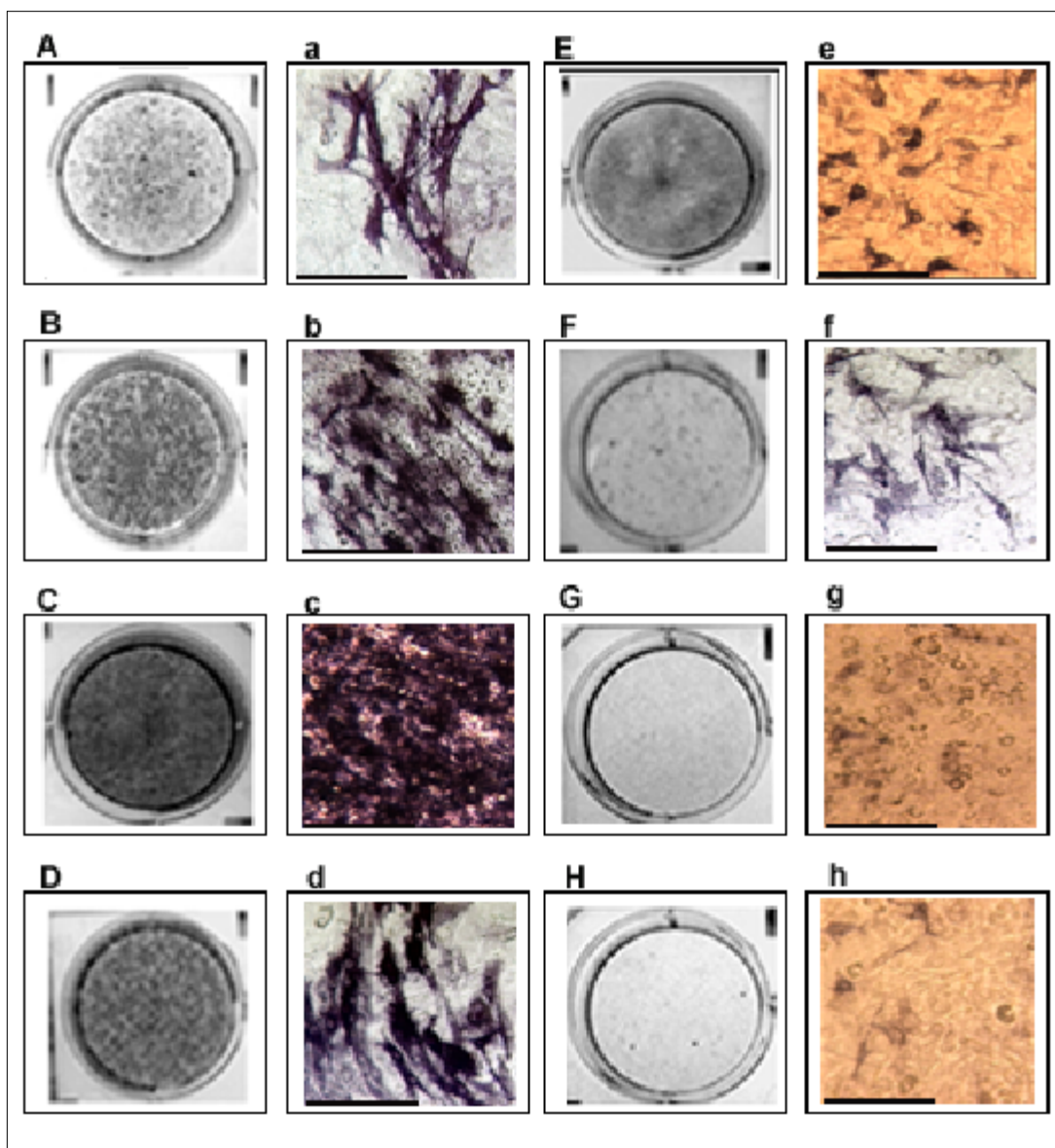
**B, b:** C3H10T1/2-Smad1 wt ;

**C, c:** C3H10T1/2-Smad1 (C+P) ;

**D, d:** C3H10T1/2-Smad1 C;



**E, e:** C3H10T1/2-Smad1 (N+P);  
**F, f:** C3H10T1/2-Smad1 N;  
**G, g:** C3H10T1/2-Smad1 (C+N);  
**H, h:** C3H10T1/2-Smad1 P;



**Fig. 2.10:** Osteoblast-like cells identified by alkaline phosphatase staining of C3H10T1/2<sub>BMP2</sub> cells expressing recombinant Smad1 or Smad1 domains (10 day post-confluence). — 100  $\mu$ m.

**A, a:** C3H10T1/2<sub>BMP2</sub> ;  
**B, b:** C3H10T1/2<sub>BMP2</sub>-Smad1 wt;  
**C, c:** C3H10T1/2<sub>BMP2</sub>-Smad1 (C+P);

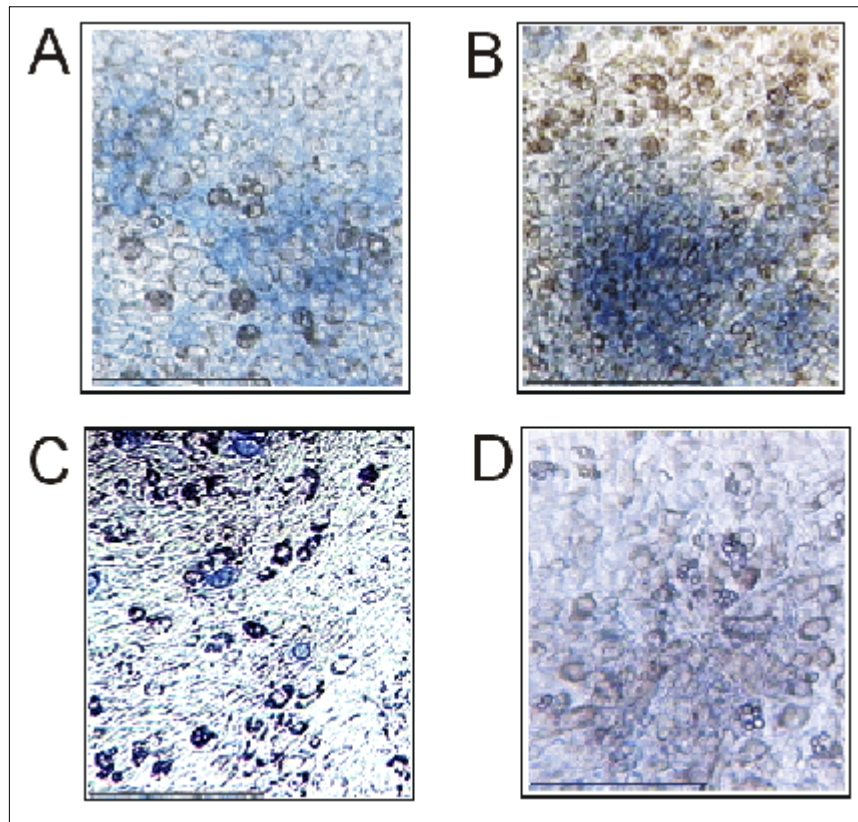
- D, d:** C3H10T1/2<sub>BMP2</sub>-Smad1 C;  
**E, e:** C3H10T1/2<sub>BMP2</sub>-Smad1 (N+P);  
**F, f:** C3H10T1/2<sub>BMP2</sub>-Smad1 N;  
**G, g:** C3H10T1/2<sub>BMP2</sub>-Smad1 (C+N);  
**H, h:** C3H10T1/2<sub>BMP2</sub>-Smad1 P;

The recombinant expression of Smad1, Smad1 (C+P) or Smad1 C domains in C3H10T1/2<sub>BMP2</sub> cells leads to enhanced levels of alkaline phosphatase positive osteoblast-like cells (Fig. 2.10. C, c; D, d). Expression of the Smad1 N domain or (N+P) domain suppressed the BMP2-dependent alkaline phosphatase activity (Fig. 2.10. F,f; E,e). Also, the typical alkaline phosphatase staining cells were not observed in C3H10T1/2<sub>BMP2</sub>- Smad1 (C+N) cells and C3H10T1/2<sub>BMP2</sub>- Smad1 P cells (Fig. 2.10. G, g, H, h).

Summarizing, the Smad1 (C+P) domain and the Smad1 C domain have the potential to constitutively induce or stimulate the differentiation of C3H10T1/2 or C3H10T1/2<sub>BMP2</sub> cells into osteoblast-like cells. The result supports the conclusion obtained in both the *Xenopus* animal cap assay and a Hela (Mv1Lu) one-hybrid assay, that the C domain is the biological active domain (Meersseman et al. 1997). In addition, the Smad1 P domain has for the first time been observed as a negative regulator for the BMP2-dependent formation of osteoblast-like cells.

### 2.2.2 Chondrocytes identified by Alcian Blue staining in cells expressing recombinant full-length Smad1 or Smad1 domains

Whether the recombinant Smad1 domains have the ability to initiate or stimulate the differentiation of C3H10T1/2 cells or C3H10T1/2<sub>BMP2</sub> cells into chondrocytes was investigated by Alcian Blue staining. Alcian Blue specifically stains the typical acid\_proteoglycans of cartilage. At the indicated time intervals fixed cells were stained with Alcian Blue at pH 2.5 overnight, washed with PBS, and then documented by light microscopy.



**Fig. 2.11:** Chondrocytes identified by Alcian Blue staining of C3H10T1/2<sub>BMP2</sub> cells expressing recombinant Smad1 wild type, Smad1 C domain, and Smad1 P domain (13 day post-confluence).

- A: C3H10T1/2<sub>BMP2</sub> cells.
- B: C3H10T1/2<sub>BMP2</sub>-Smad1 wt
- C: C3H10T1/2<sub>BMP2</sub>-Smad1 C
- D: C3H10T1/2<sub>BMP2</sub>-Smad1 P
- 100  $\mu$ m.

Alcian Blue positive cells were not observed in parental C3H10T1/2 cells. Also the C3H10T1/2 cells expressing recombinant Smad1 domains did not give rise to histologically verified chondrocytes (data not shown). However, Alcian Blue positive cells were identified in C3H10T1/2<sub>BMP2</sub> cells, C3H10T1/2<sub>BMP2</sub>-Smad1 wt cells, C3H10T1/2<sub>BMP2</sub>-Smad1 C cells, and C3H10T1/2<sub>BMP2</sub>-Smad1 P cells (Fig. 2.11. A, B, C, D). Interestingly, it seems that the chondrogenic differentiation potential is neither increased by the recombinant expression of

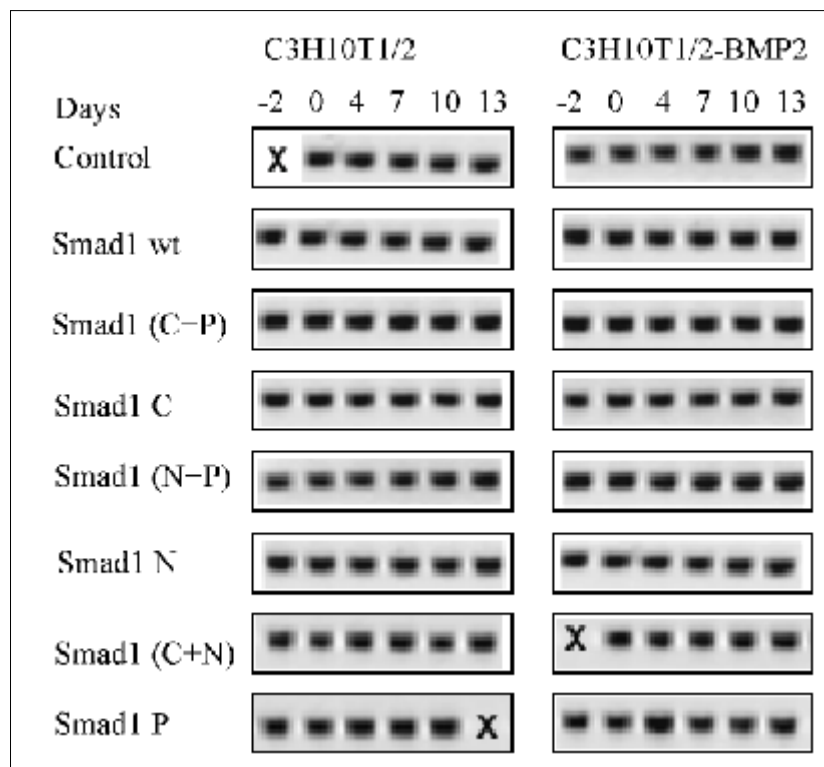


either full-length Smad1 or its biologically active domain (C domain) nor inhibited by the recombinant expression of the Smad1 P domain, which abolished the positive alkaline phosphatase staining in C3H10T1/2<sub>BMP2</sub> cells.

### 2.3 The expression of marker genes for osteogenesis, chondrogenesis and adipogenesis in cells recombinantly expressing Smad1 or Smad1 domains

The osteogenic differentiation potential resulting from recombinant expression of Smad1 and Smad1 domains was investigated histologically in C3H10T1/2 cells and C3H10T1/2<sub>BMP2</sub> cells (Chapter 2.2.1 and 2.2.2). These results were substantiated by genetic analyses of marker genes typical of or specific for osteogenesis, including the parathyroid hormone receptor, collagen I, osteopontin, osteonectin, osteocalcin and the osteogenic transcription factor CBFA1, in the cells expressing Smad1 domains. In addition, the expression of the chondrogenic marker gene collagen II and of fibroblast growth factor (FGF) receptor 3, as well as expression of the adipogenic marker gene adipoQ was investigated in our experiments. Cells (C3H10T1/2 cells and C3H10T1/2<sub>BMP2</sub> cells recombinantly expressing Smad1 and Smad1 domains) were plated at a density of 7500 cells/cm<sup>2</sup> in Roux flasks. After reaching confluence the cells were cultivated in differentiation medium (DMEM containing 10% FCS, G418 with or without Puromycin, 50 µg/ml ascorbic acid and 10 mM β-glycerophosphate), which was changed every other day. The cells were harvested at definite time intervals (2 days before and 0, 4, 7, 10, 13 days after reaching confluence) and total RNA was isolated by TRI-REAGENT<sup>LS</sup> (Chapter 5.5.7). 5 µg of total RNA was used for cDNA-synthesis (Chapter 5.4.1).

RT-PCR experiments for all cell lines were performed with primer for the housekeeping gene HPRT (hypoxanthin-guanin-phosphoribosyl-transferase) in order to equalize the amount of cDNA of different probes (Fig. 2.12). The amount of cDNA used for the subsequent PCRs for marker genes was adjusted with the computer program-PHORETIX.

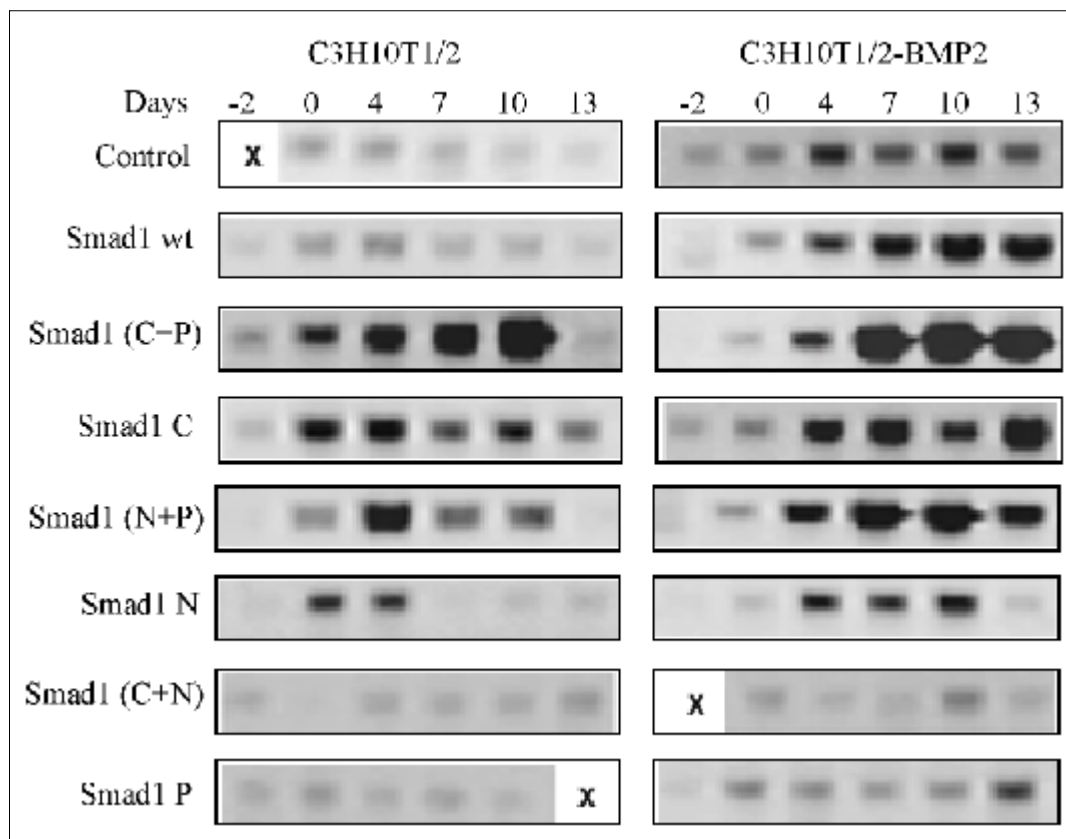


**Fig. 2.12:** Expression of HPRT in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells recombinantly expressing Smad1 or Smad1 domains (after equalization of cDNAs). 0.5 µl 1:10 cDNA stock solution, 30 cycles at 55°C. x = RNA pellet was lost during isolation.

### 2.3.1 Expression of the osteocalcin gene in cells recombinantly expressing Smad1 or Smad1 domains

Osteocalcin is a small (49 amino acids) Ca<sup>2+</sup>-binding protein. It is distinguished by its content of three gamma-carboxyglutamic (Gla) residues. The name osteocalcin derives from the abundance of this protein in bone tissue (10-20% of the noncollagen protein), and its affinity for Ca<sup>2+</sup> (Hauschka and Gallop 1977). Osteocalcin appears to be a highly specific osteoblast marker during bone formation (reviewed by Hauschka 1986). The osteocalcin transcription is regulated by different factors, for example, Vitamin A- and D- receptors, and the jun-fos complex (AP-1) (Schüle et al. 1990) .

RT-PCR with specific primers (Appendix 1: RT-Osteocalcin) was used to monitor the expression of the osteocalcin gene in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells recombinantly expressing Smad1 and Smad1 domains.



**Fig. 2.13:** RT-PCR analyses of the expression of osteocalcin gene in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells recombinantly expressing Smad1 and Smad1 domains (after equalization of cDNAs). 1 µl 1:10 cDNA stock solution, 29 cycles at 57°C. x = RNA was lost during isolation.

In C3H10T1/2 control cells the osteocalcin mRNA is present at only low levels. Whereas in the control C3H10T1/2<sub>BMP2</sub> cells the osteocalcin mRNA level is up-regulated after confluence, the maximum expression of osteocalcin in the control C3H10T1/2<sub>BMP2</sub> cells was observed at day 10 post-confluence. The Smad1 (C+P) domain, C domain and (N+P) domain stimulated the osteocalcin expression in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells. The C3H10T1/2-Smad1 N cells also showed a transient stimulation of osteocalcin gene expression. Repression of

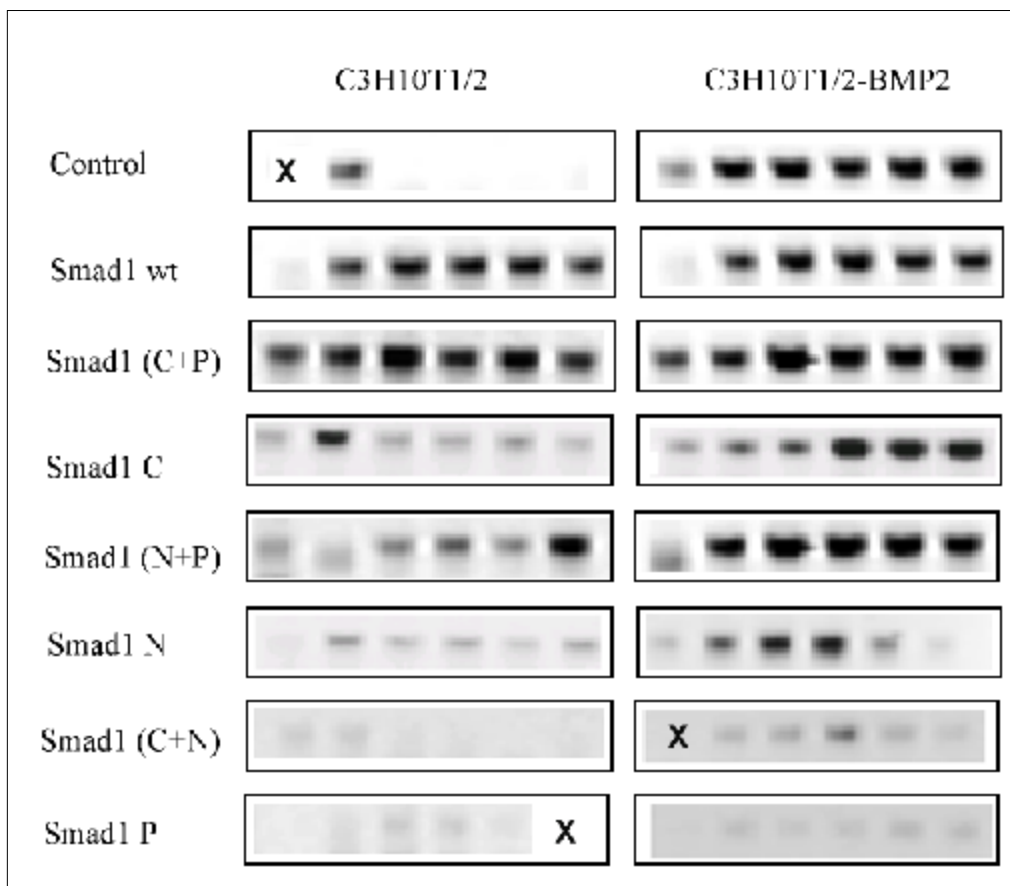
osteocalcin expression was found in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells expressing Smad1 (C+N) domain and P domain (Fig. 2.13).

So, osteocalcin mRNA transcription in parental C3H10T1/2 cells is initiated by Smad1 C and N contained domains, while the Smad1 P domain repressed osteocalcin expression in C3H10T1/2<sub>BMP2</sub> cells.

### **2.3.2 Expression of PTH/PTHrP-receptor gene in cells recombinantly expressing Smad1 or Smad1 domains**

The endocrine Parathyroid hormone (PTH) is synthesized by the parathyroid gland and has been known for decades as a crucial regulator of blood calcium. By causing bone resorption, renal tubular calcium reabsorption, and activation of vitamin D, PTH raises blood calcium levels (Chattopadhyay et al. 1996). Many of the actions of PTH and its paracrine analog PTHrP (PTH-related protein) are mediated by a PTH/PTHrP receptor, a G-coupled receptor containing seven transmembrane domains (Jüppner et al. 1991). PTH/PTHrP receptor binds and responds equally to N-terminal fragments both of PTH and PTHrP (Kronenberg et al. 1997). The PTH/PTHrP receptor mRNA is expressed in the PTH target organs, kidney and bone (Urena et al. 1993). Moreover, the PTH/PTHrP receptor is found on osteoblasts but not on osteoclasts (reviewed by Kronenberg 1997).

The expression of PTH/PTHrP receptor in cells recombinantly expressing Smad1 or its domains was investigated by RT-PCR using appropriate primers (Appendix1: RT-PTH/PTHrP R).



**Fig. 2.14:** RT-PCR analyses of the expression of PTH/PTHrP receptor gene in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells recombinantly expressing Smad1 or Smad1 domains (after equalization of cDNAs). 1 µl 1:10 cDNA stock solution, 30 cycles at 58°C. x = RNA was lost during isolation.

The PTH/PTHrP receptor gene in control C3H10T1/2 cells was hardly detected by RT-PCR, while in C3H10T1/2<sub>BMP2</sub> cells this receptor was expressed during the entire cultivation. The Smad1 wild type, the C domain, and (C+P) domain are able to stimulate the expression of PTH/PTHrP receptor in both C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells. Interestingly, the N domain and (N+P) domain are also able to increase the level of PTH/PTHrP receptor transcription in parental C3H10T1/2 cells. However, in C3H10T1/2<sub>BMP2</sub> cells the recombinant expression of N domain seemed inhibitory and reduced the level of BMP2-dependent receptor transcription. The expression of PTH/PTHrP receptor gene was completely inhibited in

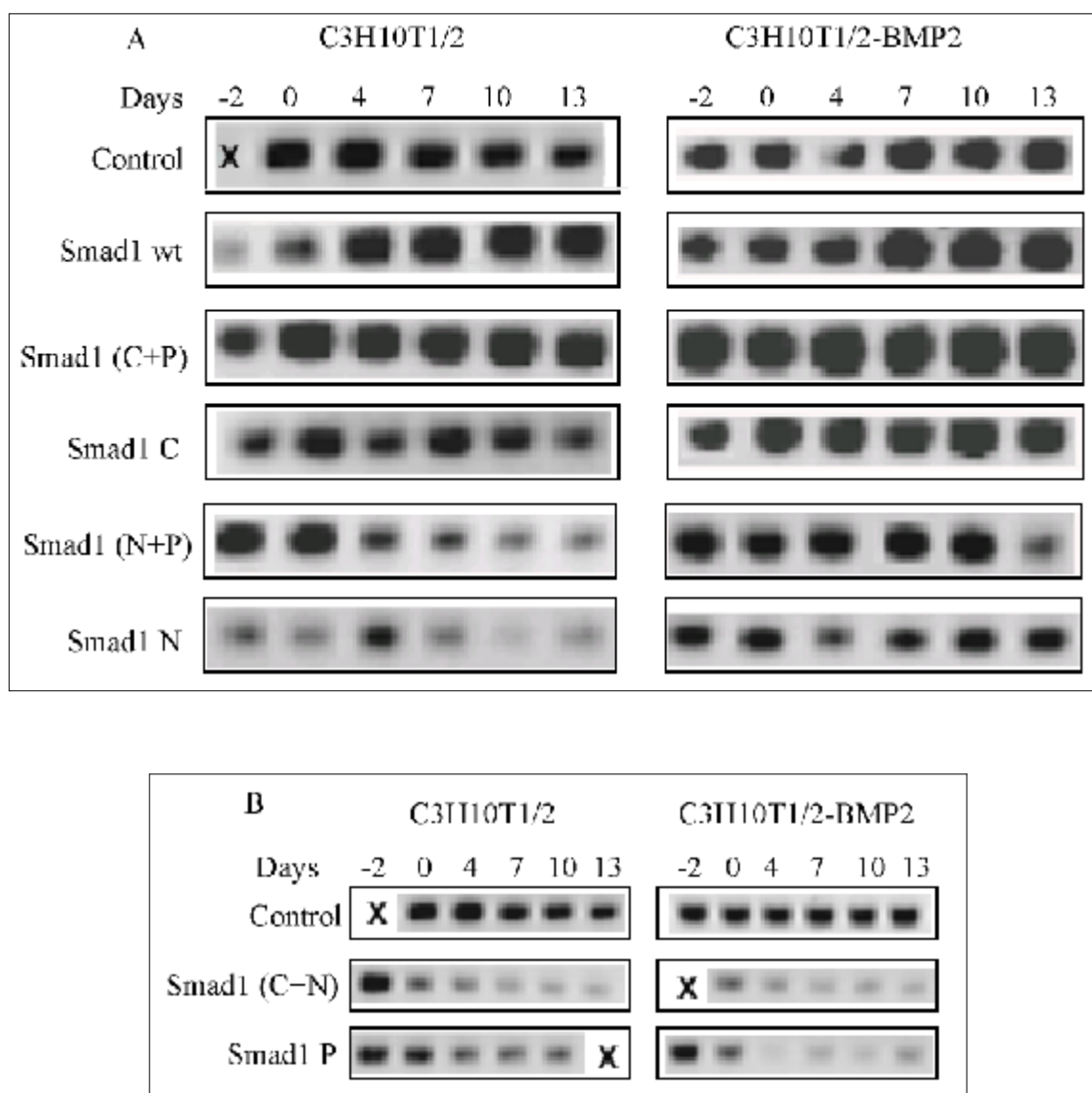
C3H10T1/2 cells and C3H10T1/2<sub>BMP2</sub> cells expressing Smad1 (C+N) domain or Smad1 P domain (Fig. 2.14).

### **2.3.3 Expression of collagen I gene in cells expressing recombinant Smad1 or Smad1 domains**

In addition to the PTH/PTHrP receptor gene, the collagen I gene is another early regulated gene in the osteogenic development. Collagen I is the major extracellular matrix protein of bone, constituting about 90% of the total organic matrix in mature bone. Collagen I is a primary product of osteoblasts during bone formation, and it is a characteristic marker for the osteoblast phenotype (Heersche et al. 1992; Helder et al. 1993).

By RT-PCR and a set of specific primers (Appendix 1: RT-Collagen I) the expression of the collagen I gene in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells expressing recombinant Smad1 or Smad1 domains was investigated.

The RT-PCR analyses showed that collagen I specific mRNA is expressed in the fibroblastic parental C3H10T1/2 cells. The wild type Smad1 enhanced the expression of the collagen I in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells. The collagen I mRNA levels were considerably enhanced in C3H10T1/2-Smad1 C, C3H10T1/2-Smad1 (C+P), C3H10T1/2<sub>BMP2</sub>-Smad1 C, and C3H10T1/2<sub>BMP2</sub>-Smad1 (C+P) cells (Fig. 2.15 A). The Smad1 (N+P) domain and Smad1 N domain caused a repression of the expression of the collagen I gene in C3H10T1/2 cells. An inhibition of the expression of collagen I was also observed in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells expressing Smad1 (C+N) domain and P domain (Fig. 2.15 B).



**Fig. 2.15:** RT-PCR analyses of the expression of collagen I gene in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells recombinantly expressing Smad1 or its domains (after equalization of cDNAs). 0.8 µl 1:10 cDNA stock solution, 29 cycles at 50°C. x = RNA was lost during isolation.

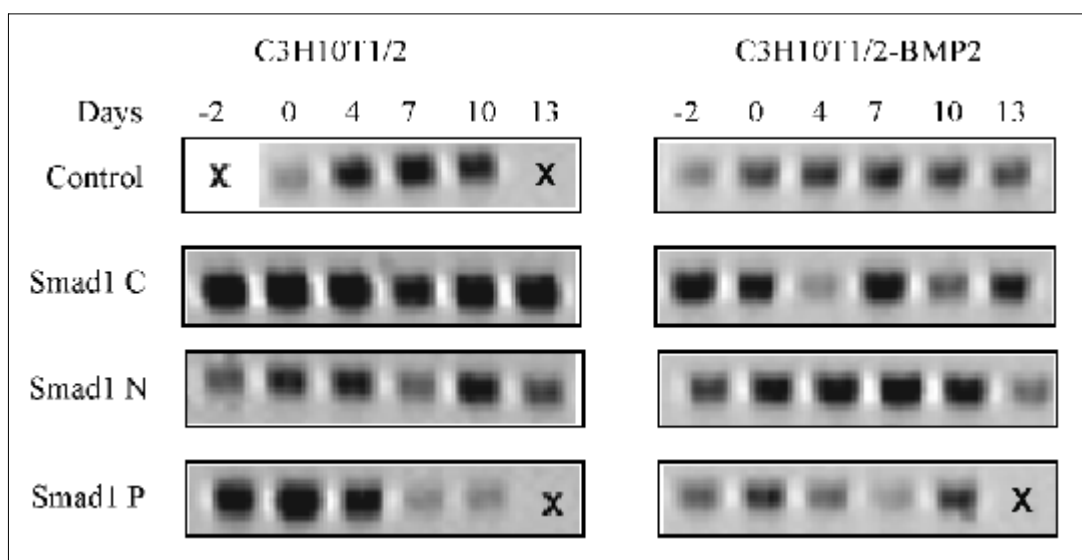
#### 2.3.4 The recombinant expression of *CBFA1* gene in C3H10T1/2 cells is not significantly regulated by Smad1 or Smad1 domains



CBFA1, also called  $\text{Pebp}\alpha 2\text{A}$  (polyoma enhancer-binding protein), is a member of the core-binding factor (CBF) transcription factors. CBFA1 has a DNA binding domain, *runt*, which is homologous to the *Drosophila* pair-rule gene *runt* (Kania et al. 1990). CBFA1 was recently shown to interact with the promoter region of the osteocalcin gene and play an essential role in osteoblast differentiation (Ducy et al. 1997).

CBF transcription factors are a family of heterodimeric proteins having two unrelated subunits: a DNA binding  $\alpha$  subunit and a non-DNA binding  $\beta$  subunit. The mammalian CBF  $\alpha$  subunits are encoded by three distinct genes (*CBFA1*, *CBFA2*, and *CBFA3*) (Ito 1996), and a common  $\beta$  subunit is encoded by the *CBFB* gene (reviewed by Speck and Terry 1995).

With a set of specific primers which were located in the specific NH<sub>2</sub>-terminal of the CBFA1 (Appendix 1: RT-CBFA1), the expression of *CBFA1* gene was investigated in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells expressing recombinant Smad1 or Smad1 domains.



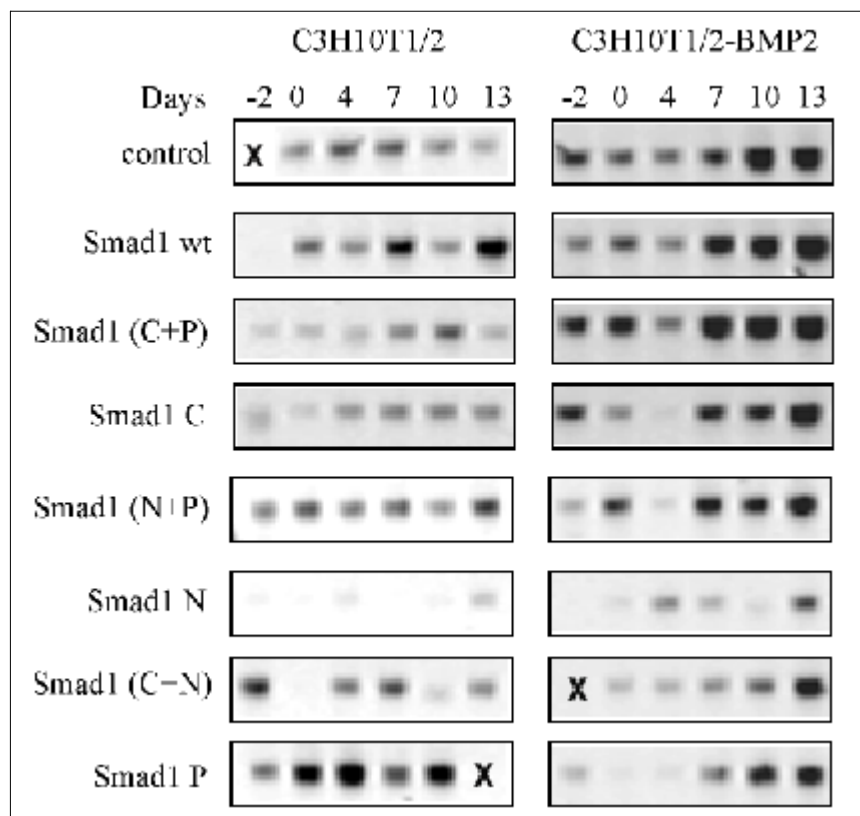
**Fig. 2.16:** RT-PCR analyses of the expression of *CBFA1* gene in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells recombinantly expressing Smad1 or Smad1 domains (after equalization of cDNAs). 1  $\mu$ l 1:10 cDNA stock solution, 35 cycles at 55°C. x = RNA was lost during isolation.

RT-PCR analyses showed the *CBFA1* to be expressed in both C3H10T1/2 cells and C3H10T1/2<sub>BMP2</sub> cells, but BMP2 background did not enhance its expression compared to parental C3H10T1/2 cells. The Smad1 C domain, N domain and P domain did not affect the *cbfa1* expression either in C3H10T1/2 cells or in C3H10T1/2<sub>BMP2</sub> cells (Fig. 2.16). These results are consistent with the observations by Noda and colleagues, who have demonstrated that the BMP4/7 heterodimer enhances *cbfa1* expression, whereas BMP2 does not enhance its expression in MC3T3E1 and C2C12 cells (Tsuji et al. 1998). If BMP2 itself does not regulate the expression of *CBFA1*, it is conclusive that its signaling mediator Smad1 has no influence on *CBFA1* expression.

### **2.3.5 Expression of osteopontin gene in cells recombinantly expressing Smad1 or Smad1 domains**

Osteopontin is a glycosylated phosphoprotein with a high number of acidic amino acid residues and constitutes an abundant protein produced by osteoblasts (Mark et al. 1987). It is expressed along with osteocalcin in relatively late developmental stages (Owen et al. 1990). Osteopontin has also been observed in various cells, including epidermal cells, kidney, placenta, neuronal tissue, some cell types of the lymphopoietic system, and chondrocytes, although, at far lower expression levels (Ahrens et al. 1993). Osteopontin expression is another strong indication for the osteogenic developmental sequence because it is second only to osteocalcin in the extent and depth of bone matrix immuno staining (Carlson et al. 1993).

RT-PCR with a set of specific primers (Appendix 1: RT-Osteopontin ) enabled monitoring of the expression of the osteopontin gene in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells expressing recombinant Smad1 or Smad1 domains (Fig. 2.17).



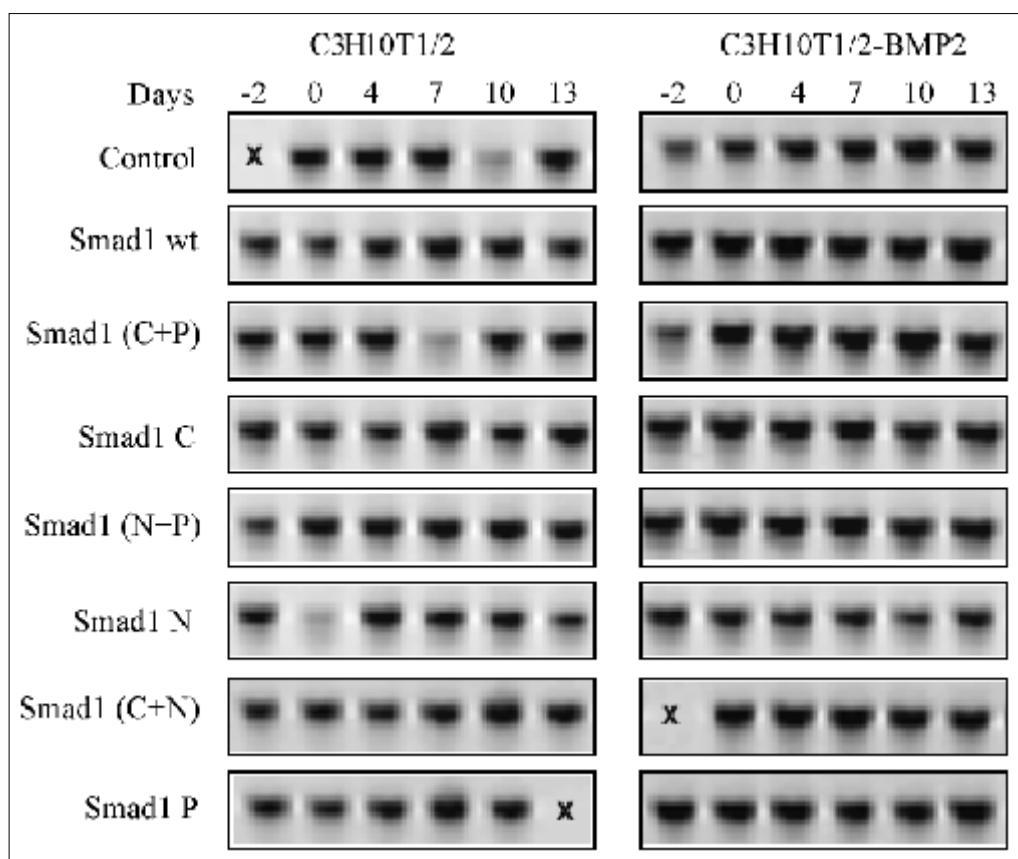
**Fig. 2.17:** RT-PCR analyses of the expression of osteopontin gene in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells recombinantly expressing Smad1 or Smad1 domains (after equalization of cDNAs). 1 µl 1:10 cDNA stock solution, 30 cycles at 53°C. x = RNA was lost during isolation.

The expression of osteopontin gene in control C3H10T1/2 cells was down-regulated after reaching confluence, while in control C3H10T1/2<sub>BMP2</sub> cells osteopontin expression was up-regulated after confluence (Fig. 2.17). Interestingly, Smad1 P domain increased the osteopontin mRNA levels in C3H10T1/2 cells but not in C3H10T1/2<sub>BMP2</sub> cells. The Smad1 wild type, (C+P) domain, C domain, (N+P) domain, and (C+N) domain did not change the intensity of osteopontin expression in C3H10T1/2 cells. The N domain down-regulated mRNA levels in both C3H10T1/2 cells and C3H10T1/2<sub>BMP2</sub> cells.

### 2.3.6 Expression of osteonectin gene in cells recombinantly expressing Smad1 or Smad1 domains

Osteonectin is an acidic phosphoprotein that was originally found in bone matrix in high relative abundance and is virtually absent from other tissues (Termine et al. 1981). However, expression and secretion of osteonectin have also been documented in various other cell types including chondrocyte of chondroid bone, though, at a far lower level (reviewed by Tracy et al. 1988).

By RT-PCR and specific primers (Appendix 1: RT-Osteonectin) the expression of osteonectin gene in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells expressing recombinant Smad1 or Smad1 domains was investigated.



**Fig. 2.18:** RT-PCR analyses of expression of the osteonectin gene in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells recombinantly expressing Smad1 or Smad1 domains (after equalization of cDNAs). 0.8 µl 1:10 cDNA stock solution, 30 cycles at 59.7°C. x = RNA was lost during isolation.

Fig. 2.18 shows that the osteonectin gene was highly expressed in all cell lines monitored. The Smad1 domains seemed to have no clear effect on the expression of the osteonectin gene.

Thus it seems that osteopontin and osteonectin are only marginally (osteopontin) or not at all (osteonectin) influenced by Smad1 or Smad1 domains.

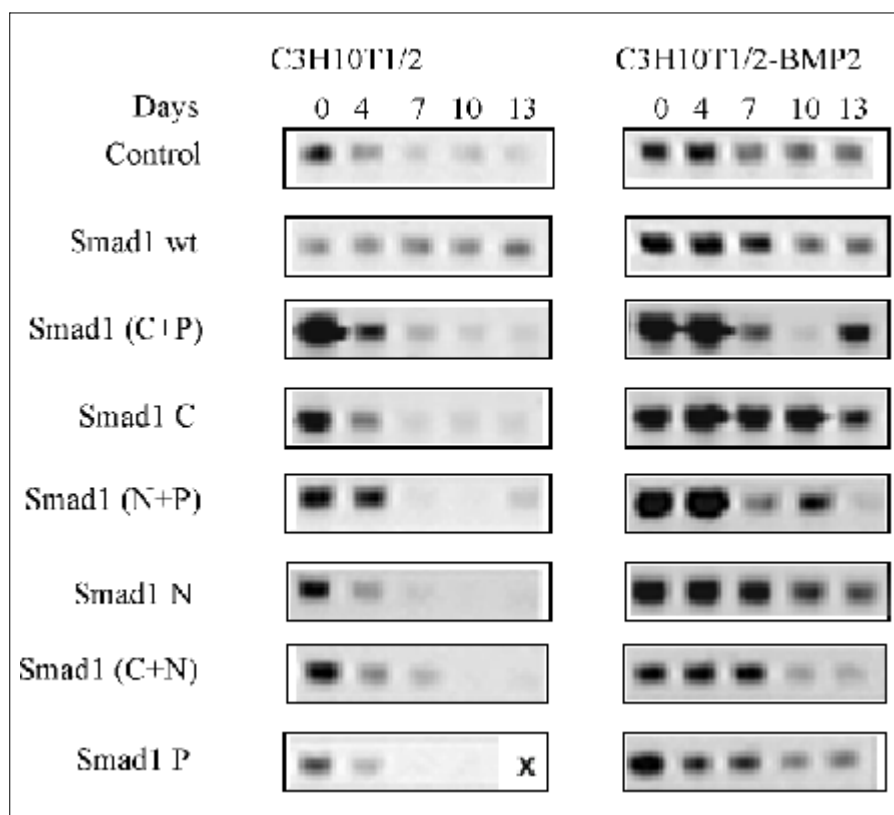
### **2.3.7 Expression of the collagen II gene in cells recombinantly expressing Smad1 or Smad1 domains**

Collagen type II is a homotrimer of  $\alpha$  (II), and is the principal protein of cartilage (Erlebacher et al. 1995). The expression of collagen II gene in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells recombinantly expressing Smad1 or Smad1 domains was investigated with the help of specific primers (Appendix 1: RT-Collagen II) by RT-PCR.

In the early cultivation stage the expression of collagen II can only marginally be detected in control C3H10T1/2 cells by RT-PCR and is downregulated after day 4 post-confluence (Fig. 2.19). The wild type Smad1 did not significantly alter the expression of the collagen II gene in C3H10T1/2 cells. Also, the Smad1 (C+P) domain, C domain, (N+P) domain, N domain, (C+N) domain and P domain did not significantly change the expression of collagen II gene in C3H10T1/2 cells.

The expression of collagen II in C3H10T1/2<sub>BMP2</sub> cells was detected during the entire cultivation. Again the Smad1 wild type had no influence on collagen II expression levels. The Smad1 C domain seemed to stimulate the expression of the collagen II gene in the late cultivation stage in C3H10T1/2<sub>BMP2</sub> cells, however, this is questionable, since (C+P) domain

did not show this increase. None of Smad1 N domain, (N+P) domain, (C+N) domain and P domain changed collagen II mRNA levels in C3H10T1/2<sub>BMP2</sub> cells (Fig. 2.19).



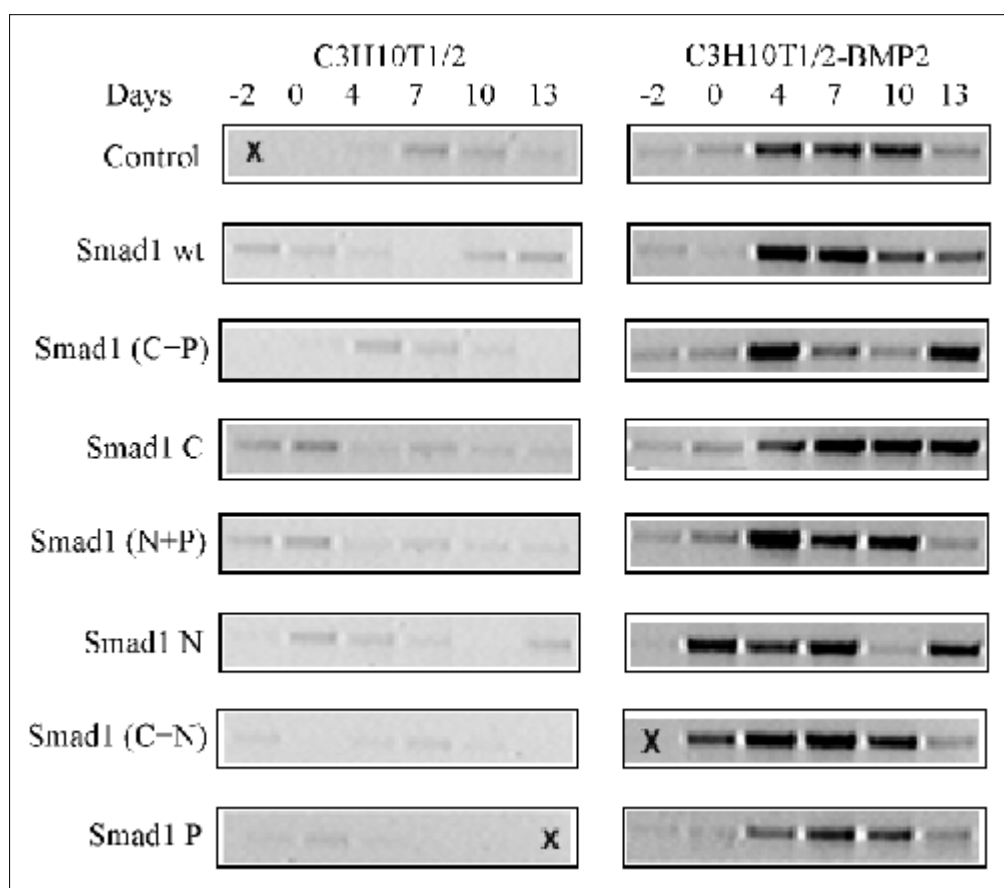
**Fig. 2.19:** RT-PCR analyses of expression of the collagen II gene in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells recombinantly expressing Smad1 or Smad1 domains (after equalization of cDNAs). 1 µl 1:10 cDNA stock solution, 30 cycles at 54°C. x = RNA was lost during isolation.

The RT-PCR analyses showed that recombinant Smad1 and Smad1 domains have not affected the expression of collagen II gene in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells. This result suggested the BMP2-dependent stimulation of collagen II gene expression was not regulated by Smad1.

### 2.3.8 Expression of FGF receptor 3 gene in cells recombinantly expressing Smad1 or Smad1 domains

Fibroblast growth factor receptor 3 (FGFR-3) is one of four distinct membrane-spanning tyrosine kinases that serve as high affinity receptors for at least nine fibroblast growth factors (FGFRs) (reviewed by Basilico and Moscatelli 1992). FGFR-3 mRNA is expressed at high level in the cartilage rudiments of a wide variety of bones (Peters et al. 1992), suggesting a role for FGFR-3 in bone development. Deng and his colleagues showed that FGFR-3 is expressed in the proliferation zone of chondrocytes of postnatal mice, and a normal function of FGFR-3 is to limit rather than promote osteogenesis (Deng et al. 1996).

With a set of specific primers (Appendix1: FGFR-3) the expression of the FGFR-3 gene in C3H10T1/2 and C3H10T1/2<sup>BMP2</sup> cells expressing recombinant Smad1 or Smad1 domains was investigated by RT-PCR.



**Fig. 2.20:** RT-PCR analyses of the expression of the FGFR-3 gene in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells recombinantly expressing Smad1 or Smad1 domains (after equalization of cDNAs). 2 µl 1:10 cDNA stock solution, 30 cycles at 55°C. x = RNA was lost during isolation.

The expression of FGFR-3 was hardly detectable in parental C3H10T1/2 cells, C3H10T1/2-Smad1 wt cells and C3H10T1/2 cells expressing Smad1 domains. The control C3H10T1/2<sub>BMP2</sub> cells expressed higher levels of FGFR-3 in comparison with C3H10T1/2 cells. Smad1 wild type, (C+P) domain, C domain, (N+P) domain, N domain, (C+N) domain and P domain did not change the expression of FGFR-3 in C3H10T1/2<sub>BMP2</sub> cells. (Fig. 2.20)

These RT-PCR analyses imply that BMP2 enhances the FGFR-3 gene expression of C3H10T1/2 cells, but this BMP2-dependent stimulation of the expression of FGFR-3 gene is not regulated by Smad1 or its domains.

### 2.3.9 Expression of an adipogenic marker gene, AdipoQ, in cells recombinantly expressing Smad1 or Smad1 domains

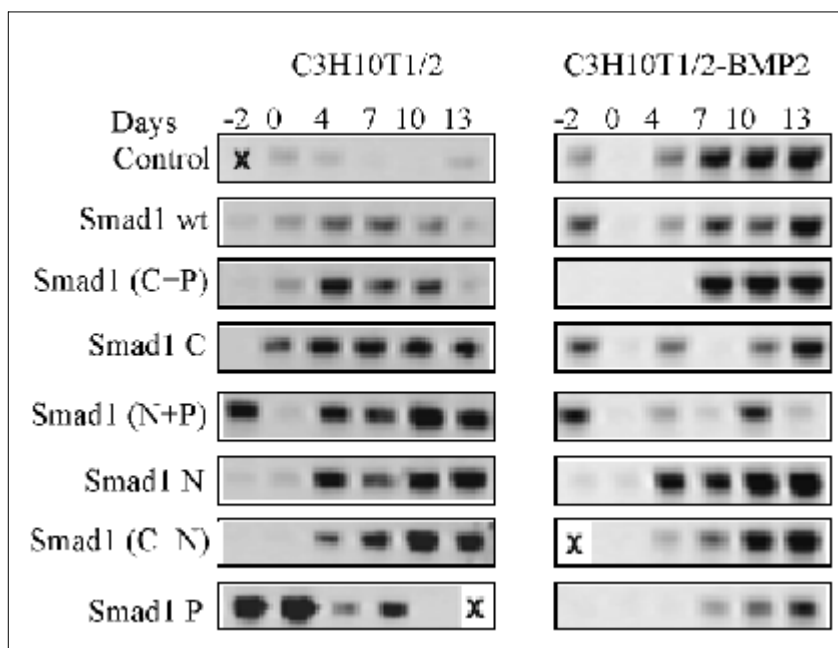
AdipoQ is a polypeptide of 247 amino acids with a secretory signal sequence at the amino terminus, a collagenous region (Gly-X-Y repeats) and a globular domain. The expression of adipoQ is highly specific for adipose tissue and is observed exclusively in mature fat cells (Hu et al. 1996).

The expression of adipoQ in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells expressing recombinant Smad1 or Smad1 domains was investigated with a set of specific primers (Appendix1: RT-adipoQ) by RT-PCR.

The expression of adipoQ in control C3H10T1/2 cells is hardly detectable (Fig. 2.21), while in C3H10T1/2 cells recombinantly expressing Smad1 and Smad1 domains adipoQ gene was expressed at increased levels. In control C3H10T1/2<sub>BMP2</sub> cells adipoQ was highly expressed at the late stages of cultivation (days 7-13 post-confluence). Smad1 wt, (C+P) domain, N domain



and (C+N) domain did not change the expression of adipoQ in C3H10T1/2<sub>BMP2</sub> cells. The adipoQ gene was weakly expressed in Smad1 C domain, (N+P) domain and P domain transfected C3H10T1/2<sub>BMP2</sub> cells, however, this is suspicious, because the (C+P) domain and N domain did not affect adipoQ expression (Fig. 2.21).

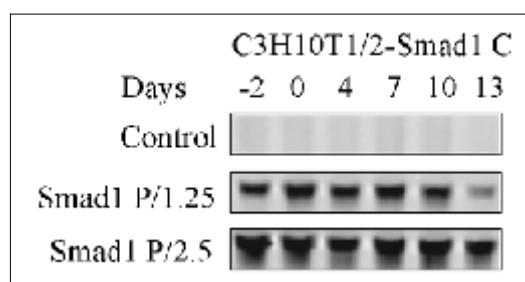


**Fig. 2.21:** RT-PCR analyses of the expression of AdipoQ gene in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells recombinantly expressing Smad1 or Smad1 domains (after equalization of cDNAs). 1µl 1:10 cDNA stock solution, 31 cycles at 55 °C. x = RNA was lost during isolation.

The expression of adipoQ in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells expressing recombinant Smad1 or Smad1 domains leads to the conclusion that adipogenesis might be regulated by this signaling molecule, but additional factors seem to exert a great influence on the level of ongoing adipogenesis.

## 2.4 The Smad1 P domain inhibits the Smad1 C domain-dependent osteogenesis in C3H10T1/2 cells

The results presented above showed that the Smad1 P domain has inhibitory function on BMP2-dependent osteogenesis rather than chondrogenesis in C3H10T1/2 cells. This investigation was extended by demonstrating that inhibitory effects of the Smad1 P domain exist on Smad1 C domain-induced osteogenesis. The Smad1 P domain was transfected in a dose-dependent manner (1.25 µg/transfection and 2.5 µg/transfection) into C3H10T1/2-Smad1 C cells. The transfections were carried out in 12-well-plates, pBS pAC (de la Luna 1988) was used as selection plasmid. Single clones were picked. Positive clones were cultivated for 13 days. Fig. 2.22 showed the recombinant Smad1 P domain to be expressed in Smad1 P/1.25 and Smad1 P/2.5 transfected C3H10T1/2-Smad1 C cells by demonstrating synthesis of the respective mRNA. In control cells no signal was detected with the primers which are specific for *Xenopus* Smad1 P domain.



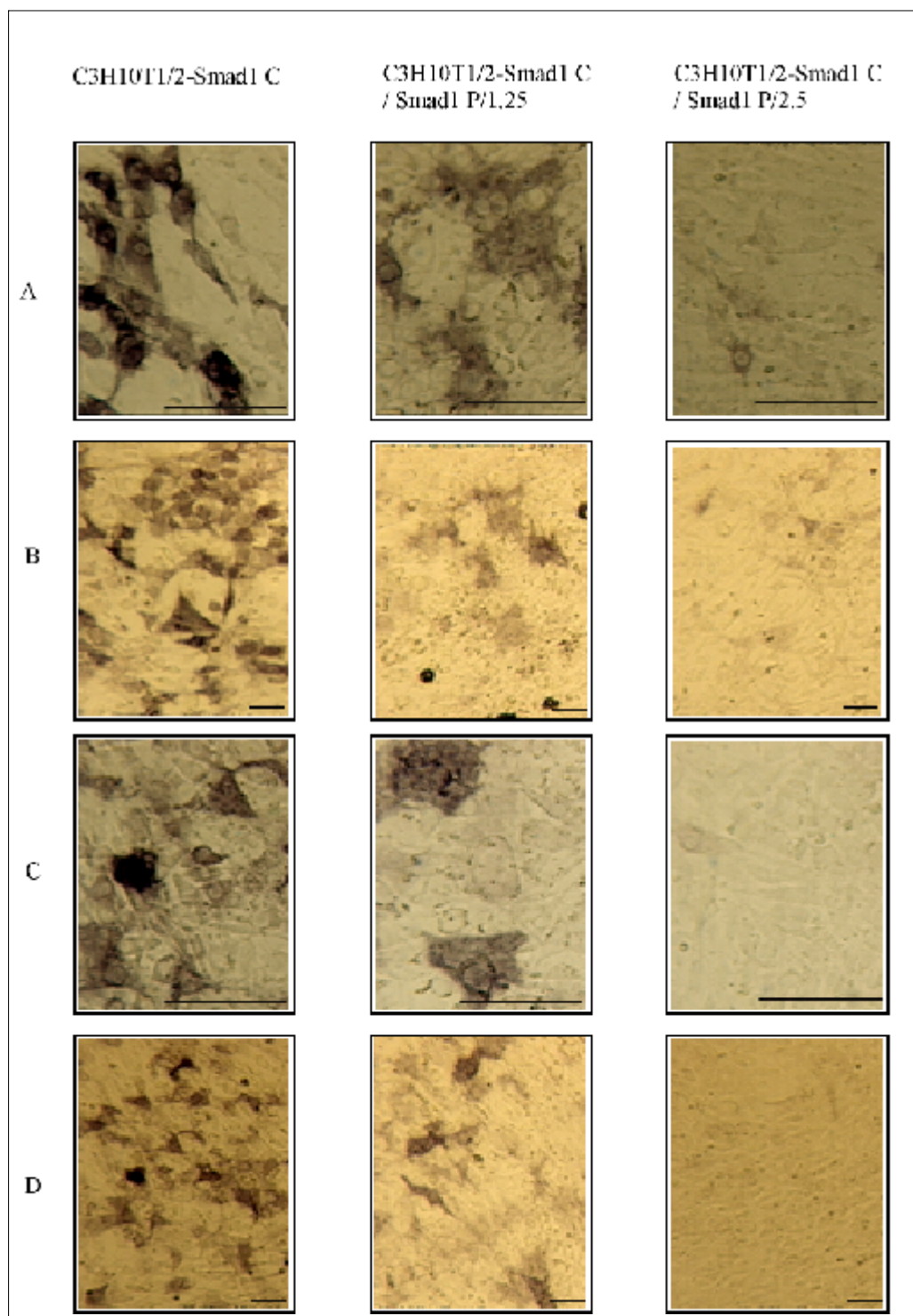
**Fig. 2.22:** Recombinant expression of Smad1 P domain in C3H10T1/2-Smad1 C cells by RT-PCR.

Smad1 P /1.25 = 1.25 µg Smad1 P domain / transfection in a well of 12-well-plate

Smad1 P /2.5 = 2.5 µg Smad1 P domain / transfection in a well of 12-well-plate

### 2.4.1 The Smad1 P domain inhibits Smad1 C domain-dependent formation of osteoblast-like cells in C3H10T1/2

The alkaline phosphatase activity was investigated in Smad1 P domain transfected C3H10T1/2-Smad1 C cells by staining with Sigma Fast BCIP/NBT tablets (Chapter 5.5.9.1).



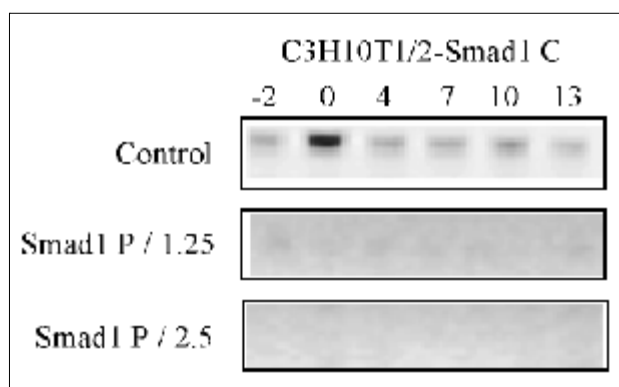
**Fig. 2.23:** Alkaline phosphatase staining of C3H10T1/2-Smad1 C cells recombinantly expressing Smad1 P domain. \_\_\_\_\_ 100  $\mu$ m.

- A: Day 13 post-confluence; 275-fold magnification
- B: Day 13 post-confluence; 85-fold magnification
- C: Day 7 post-confluence; 275-fold magnification
- D: Day 7 post-confluence; 85-fold magnification

Expression of the Smad1 P/1.25 suppressed the alkaline phosphatase activity in C3H10T1/2-Smad1 C cells at both day 7 and day 13 post-confluence. This inhibitory influence on the alkaline phosphatase activity is even more dramatic in C3H10T1/2-Smad1 C cells expressing Smad1 P/2.5 (Fig. 2.23) suggesting that the Smad1 P domain inhibits Smad1 C-induced formation of osteoblast-like cells in a dose-dependent manner.

#### 2.4.2 The Smad1 P domain inhibits the Smad1 C domain-induced PTH/PTHrP receptor gene and osteocalcin gene expression in C3H10T1/2 cells

The Smad1 P domain down-regulates the BMP2-dependent expression of both the osteocalcin and PTH/PTHrP receptor gene (Chapter 2.3.1 and 2.3.2). The dose-dependent effect of Smad1 P domain on the PTH/PTHrP receptor and osteocalcin gene expression in C3H10T1/2-Smad1 C cells confirms this result (Fig. 2.24, 2.25).

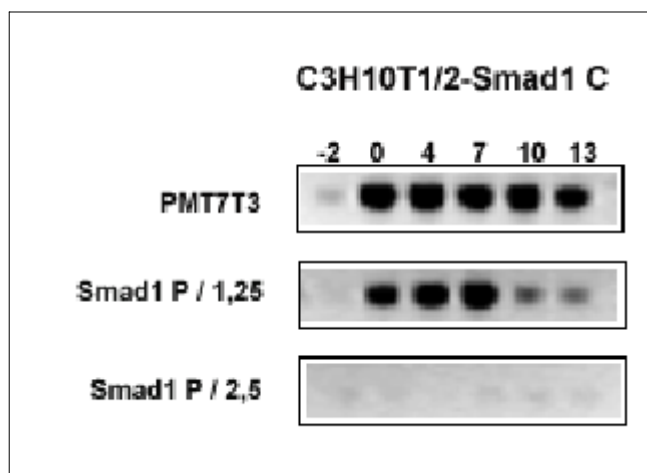


**Fig. 2.24:** RT-PCR analyses of the dose-dependent influence of Smad1 P domain on the expression of the PTH/PTHrP receptor gene in C3H10T1/2-Smad1 C cells. 1  $\mu$ l 1:10 cDNA stock solution, 30 cycles at 58°C.

Smad1 P/1.25 = 1.25  $\mu$ g Smad1 P domain/transfection in a well of 12-well-plate

Smad1 P/2.5 = 2.5 µg Smad1 P domain/transfection in a well of 12-well-plate

As shown in Fig. 2.24, expression of the PTH/PTHrP receptor gene in C3H10T1/2-Smad1 cells was inhibited by transfection with Smad1 P domain.



**Fig. 2.25:** RT-PCR analyses of the dose-dependent influence of Smad1 P domain on the expression of the osteocalcin gene in C3H10T1/2-Smad1 C cells.

Smad1 P/1.25 = 1.25 µg Smad1 P domain/transfection in a well of 12-well-plate

Smad1 P/2.5 = 2.5 µg Smad1 P domain/transfection in a well of 12-well-plate

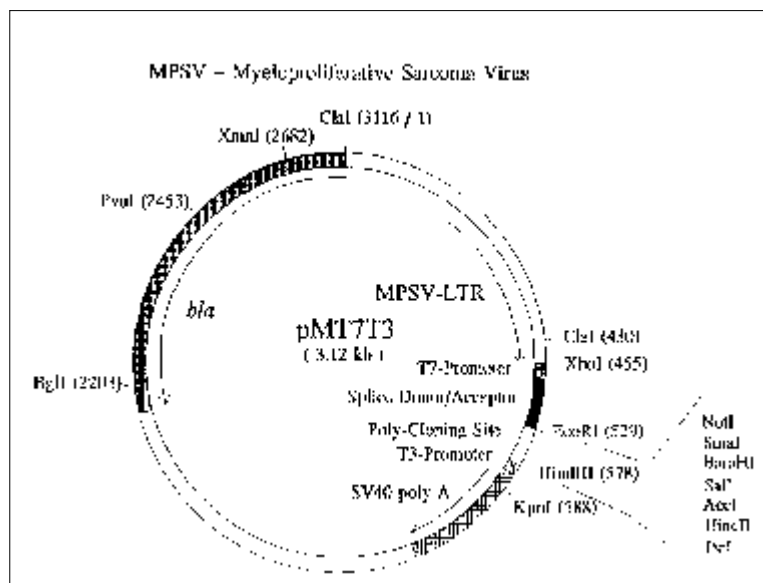
The RT-PCR analyses of expression of the PTH/PTHrP receptor gene and osteocalcin gene, combined with the alkaline phosphatase staining data, have demonstrated that the Smad1 P domain exerts a repressive effect on the Smad1 C domain-induced osteogenesis in C3H10T1/2 cells. Together with the result that BMP2 induced osteogenesis was to some extent repressed by Smad1 P domain, one can conclude that Smad1 P domain is a negative regulator of osteogenetic development in C3H10T1/2 cells.

## 2.5 The linker region of Smad3 does not affect BMP2-dependent osteogenesis in C3H10T1/2 cells

The Smad1-P domain has been demonstrated above to suppress the BMP2-dependent osteogenesis in C3H10T1/2 cells. Two questions remain to be answered regarding the possible mechanism of this repression. The first question is whether the repression of P domain on the osteogenesis is a common effect of Smads P domains or whether it is restricted to Smad1 P domain; the second is which factor might interact with Smad1 P domain to bring about the repression of the formation of osteoblast-like cells. For these reasons, the linker region of a TGF- $\beta$ /activin signal mediator, Smad3, was cloned and transfected into C3H10T1/2<sub>BMP2</sub> cells to investigate if the BMP2-dependent osteogenesis was affected by Smad3 P domain. It should be also investigated whether Smad4 is involved in the Smad1 P domain-dependent inhibition of osteogenesis.

### 2.5.1 Cloning of the linker domain of Smad3 in the eukaryotic expression vector pMT7T3

A human Smad3 cDNA clone was used to isolate the linker region by PCR and cloning primers with convenient restriction enzyme sites (*EcoRI* and *Hind* III) and an eukaryotic ribosomal binding site with ATG start codon. The PCR product was inserted into the *EcoRI/Hind* III site of the eukaryotic expression vector pMT7T3 (Fig. 2.26). The integration of the insert and the functional reading frame were verified by DNA-sequence.



**Fig. 2.26:** The eukaryotic expression vector pMT7T3

### 2.5.1.1 PCR-amplification of the linker region of Smad3

The cloning PCR was carried out by Expand<sup>TM</sup> High Fidelity PCR System, which is composed of a unique enzyme mix containing thermostable Taq DNA and Pwo DNA polymerases (Bernes et al. 1994).

↓*EcoR* I

5' TCGAATTCGCCACCATGATCCCGGCCGAGTTCCCCCACT

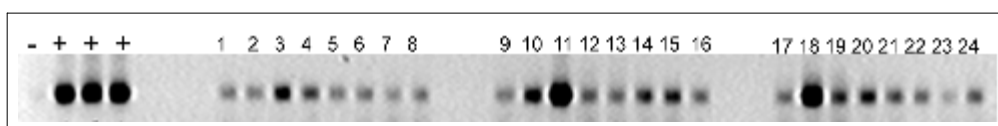
3' ATCGAAAGCTTTTTAACCGTAGGTGACTGGCTGTAG

↑*Hind* III

After amplification, the PCR product was digested with *EcoR*I/*Hind* III and inserted into the compatible restriction sites in pMT7T3.

### 2.5.1.2 Cloning of the PCR-product into pMT7T3

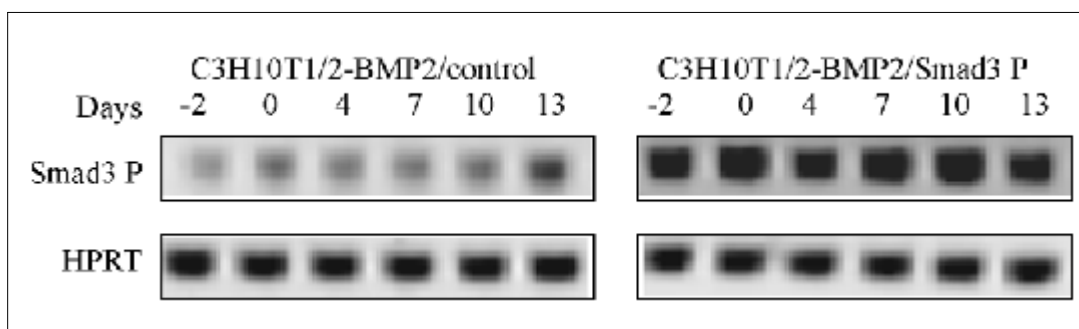
The PCR product was extracted with phenol (Chapter 5.2.6) followed by a restriction digestion with *EcoR* I and *Hind* III for 2 hrs. After separation of the fragments by running a LMP-agarose gel, the desired band was isolated from the gel (Chapter 5.2.4). The 243 bp linker region of Smad3 was then ligated with the pMT7T3 expression vector, which had been cut accordingly with *EcoR* I and *Hind* III (Chapter 5.2.1). The ligation was performed with T4 DNA ligase and incubated at 16 °C overnight (Chapter 5.3.10). 1 µl of the ligation mixture was transformed into the competent cell *E. coli* SURE (Chapter 5.3.5). After transformation, clones were tested by PCR (Chapter 5.3.6) to search for positive clones with correct inserts (Fig. 2.27).



**Fig 2.27:** Identifying the positive clones by PCR analyses for the presence of linker domain of Smad3 in the picked clons. ‘-’ = negative control; ‘+’ = positive control; number1-24 = the PCR products from different clones.

The nucleotide sequence of the insert (the linker region of SMAD3) of clone 18 was confirmed by being sequenced with ABI Prism 310 Genetic Analyzer (Chapter 5.4.4.2). Clone 18 was transfected into C3H10T1/2<sub>BMP2</sub> cells. After selection by G418, positive single clones were picked and cultivated for 13 days. Fig. 2.28 shows the Smad3 P domain was recombinantly expressed in C3H10T1/2<sub>BMP2</sub> cells.

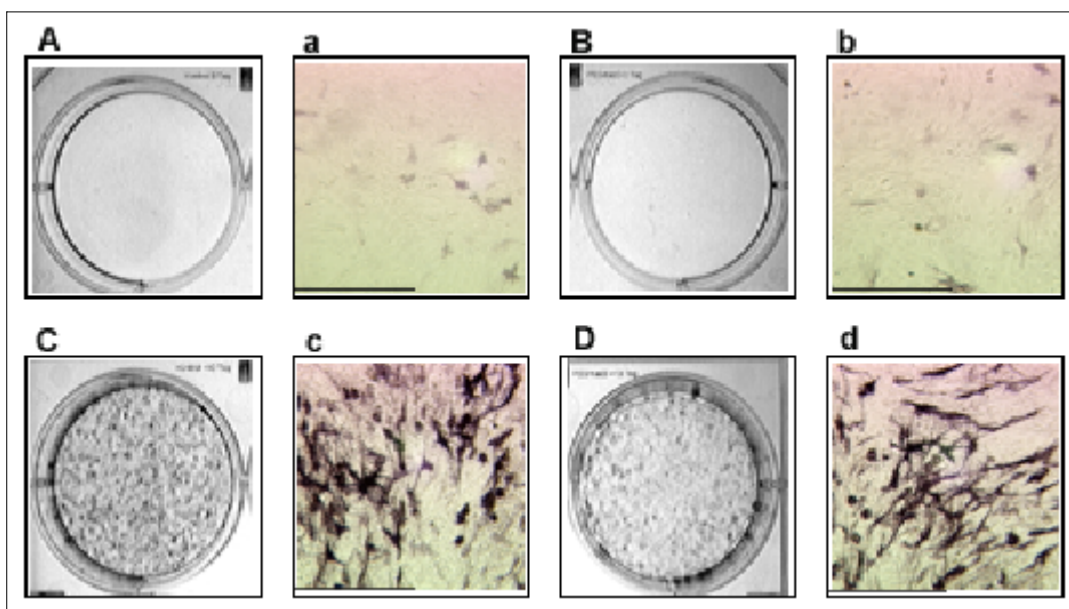




**Fig. 2.28:** RT-PCR analyses of recombinant expression of Smad3 P domain in C3H10T1/2<sub>BMP2</sub> cells.

### 2.5.2 The linker region of Smad3 does not affect the BMP2-dependent osteoblast-like cells formation in C3H10T1/2 cells

The alkaline phosphatase activity of C3H10T1/2<sub>BMP2</sub> cells expressing the linker region of Smad3 was investigated as described previously by staining with Sigma Fast BCIP/NBT tablets (Chapter 5.5.9.1).



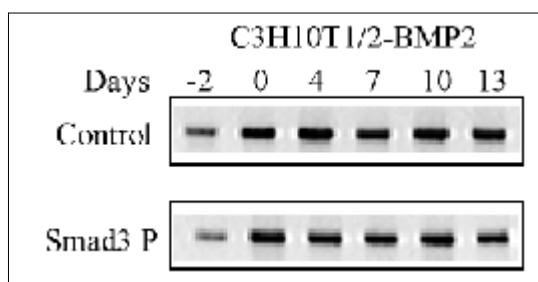
**Fig. 2.29:** Alkaline phosphatase staining of the C3H10T1/2<sub>BMP2</sub> cells expressing the linker region of Smad3.  
 — 100  $\mu$ m.

- A, a: C3H10T1/2<sub>BMP2</sub> ; day 0  
 B, b: C3H10T1/2<sub>BMP2</sub> - P domain of Smad3; day 0  
 C, c: C3H10T1/2<sub>BMP2</sub>; day 13 post-confluence  
 D, d: C3H10T1/2<sub>BMP2</sub> - P domain of Smad3; day 13 post-confluence

As shown in Fig. 2.29, the alkaline phosphatase activity of the C3H10T1/2<sub>BMP2</sub> cells was not influenced by expression of the linker region of Smad3. This result indicates that in contrast to the P domain of Smad1, the linker region of Smad3 was unable to inhibit the BMP2-dependent osteoblast-like cells formation in C3H10T1/2 cells.

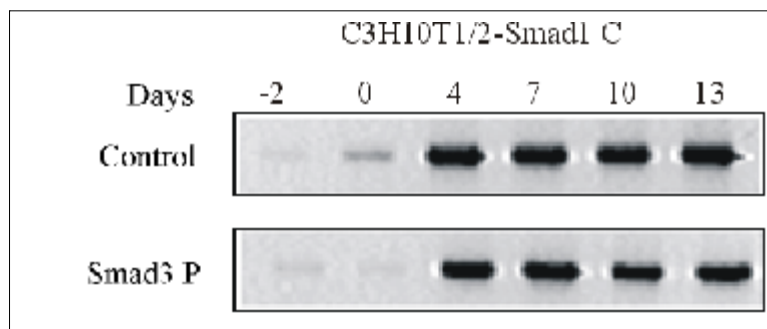
### 2.5.3 The linker region of Smad3 does not affect expression of the PTH/PTHrP receptor and osteocalcin gene

Expression of the PTH/PTHrP receptor and the osteocalcin gene was described above as being inhibited by the Smad1 P domain in C3H10T1/2 cells expressing either recombinant BMP2 or the Smad1 C domain. Whether or not the linker region of Smad3 is able to inhibit or to reduce the expression of these two osteogenic marker genes in C3H10T1/2<sub>BMP2</sub> cells was investigated by RT-PCR.



**Fig. 2.30:** RT-PCR analyses of expression of the PTH/PTHrP receptor gene in the C3H10T1/2<sub>BMP2</sub> cells expressing the linker region of Smad3 (after equalization of cDNAs). 1  $\mu$ l 1:10 cDNA stock solution, 30 cycles at 59°C.

As shown in Fig. 2.30, in C3H10T1/2<sub>BMP2</sub> cells expressing P domain (linker region) of Smad3, the PTH/PTHrP receptor mRNA level observed by RT-PCR analyses was not reduced in comparison with control C3H10T1/2<sub>BMP2</sub> cells (Fig. 2.30).



**Fig. 2.31:** RT-PCR analyses of expression of the osteocalcin in the C3H10T1/2<sub>BMP2</sub> cells expressing linker region of Smad3 (after equalization of cDNAs). 0.2 µl 1:10 cDNA stock solution, 30 cycles at 57°C.

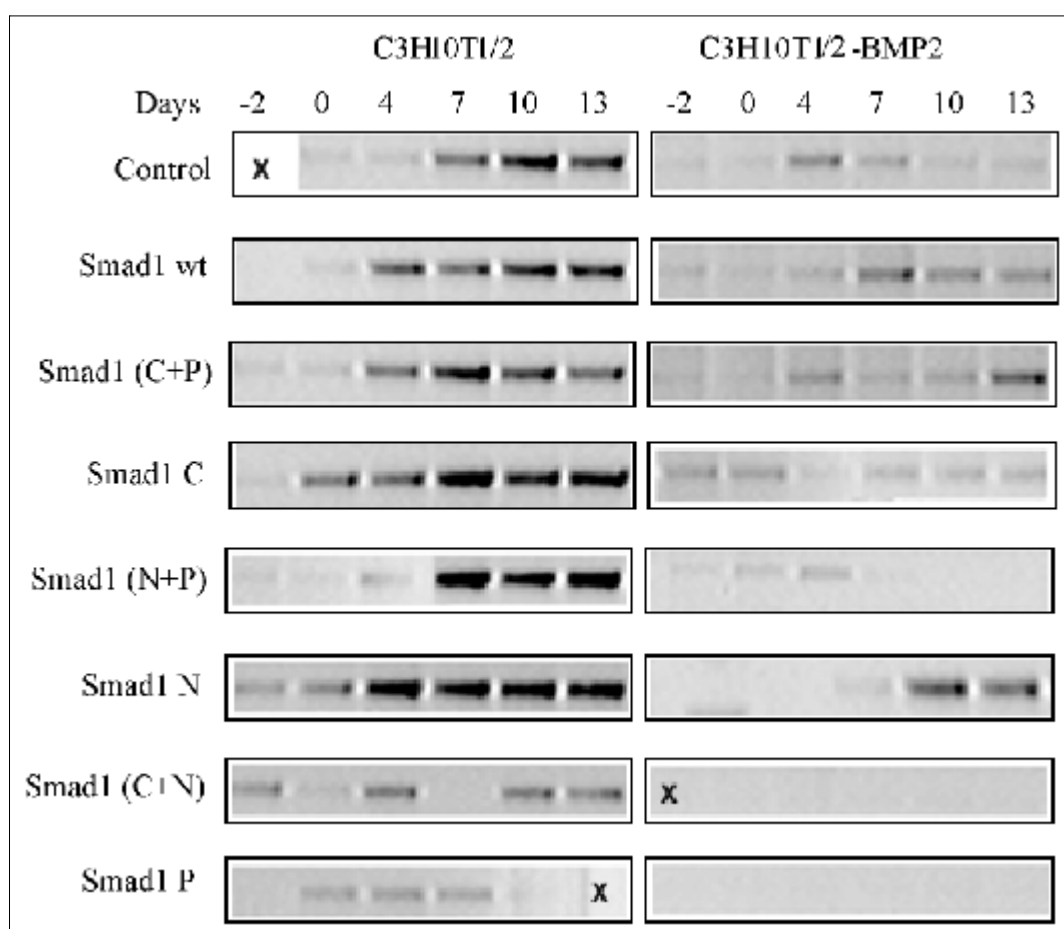
The osteocalcin gene expression in C3H10T1/2<sub>BMP2</sub> cells also was not affected by the P domain of Smad3 (Fig. 2.31). This observation, combined with the PTH/PTHrP receptor gene expression data (Fig. 2.30) and alkaline phosphatase staining data (Fig. 2.29), leads to the conclusion that the linker region of Smad3 was unable to influence the BMP2-dependent osteogenesis in C3H10T1/2 cells. Therefore, the inhibition of osteogenic development by the Smad1 P domain appears to be a specific property.

## 2.6 The Smad1 P domain mimics the BMP2-dependent down-regulation of *PEX* gene expression in C3H10T1/2 cells

PEX (phosphate regulating neutral endopeptidase on the X chromosome) is a novel neutral endopeptidase. Like other members of the neutral endopeptidase family, PEX is a type II integral membrane glycoprotein. Mutations in *PEX* underlie the phenotypic features of XLH (X-linked hypophosphatemia), which is the most common cause of heritable vitamin

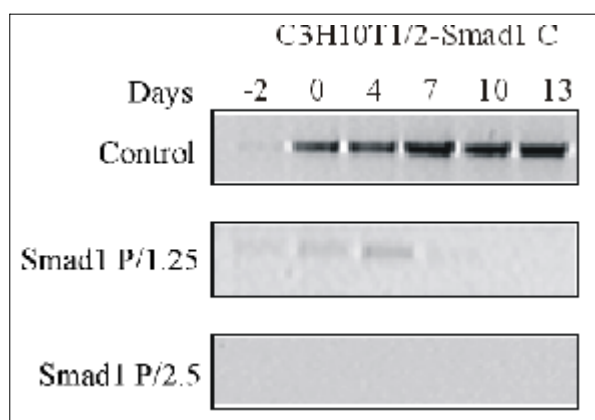
D-resistant rickets in human (Rasmussen and Tenenhouse, 1989). PEX may modulate PTH bioactivity, particularly at the level of the osteoblast, as well as the hormonal and paracrine/autocrine effects of factors produced by osteoblasts involved in regulating phosphate reabsorption and mineralization (Lipman et al. 1998).

Expression of the *PEX* gene in bone is 2-10-fold higher than in other normal tissues examined. Furthermore, PEX can effectively degrade PTH (Lipman et al. 1998). In one investigation of our lab *PEX* has been identified to be expressed in estrogen treated primary rat stroma cells and to be down-regulated by TGF- $\beta$  or BMP2 in mesenchymal progenitor C3H10T1/2 cells (Laubert 1998). Therefore, whether Smad1-signaling is involved in the BMP2-dependent down-regulation of *PEX* gene expression was investigated by RT-PCR with specific primers (Appendix 1: RT-*PEX*).



**Fig. 2.32:** RT-PCR analyses of expression of the *PEX* gene in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells recombinantly expressing Smad1 or Smad1 domains (after equalization of cDNAs). 1 µl cDNA stock solution, 30 cycles at 52.6°C. x = RNA was lost during isolation.

As mentioned above, the *PEX* gene was expressed at a higher level in C3H10T1/2 cells in comparison with in C3H10T1/2<sub>BMP2</sub> cells. Recombinant expression of Smad1 or its domains such as Smad1 wt, (C+P) domain, C domain, (N+P) domain, and N domain did not significantly influence *PEX* expression. However, BMP-mediated repression of *PEX* gene expression was mimicked in C3H10T1/2 cells by the Smad1 P domain (Fig. 2.32). Smad1 P domain is the only fragment which is able to significantly repress the *PEX* expression in C3H10T1/2 cells. The repressive effect of Smad1 P domain on *PEX* expression was confirmed by RT-PCR analyses of the C3H10T1/2-Smad1 C cells dose-dependently expressing Smad1 P domain (Fig. 2.33).

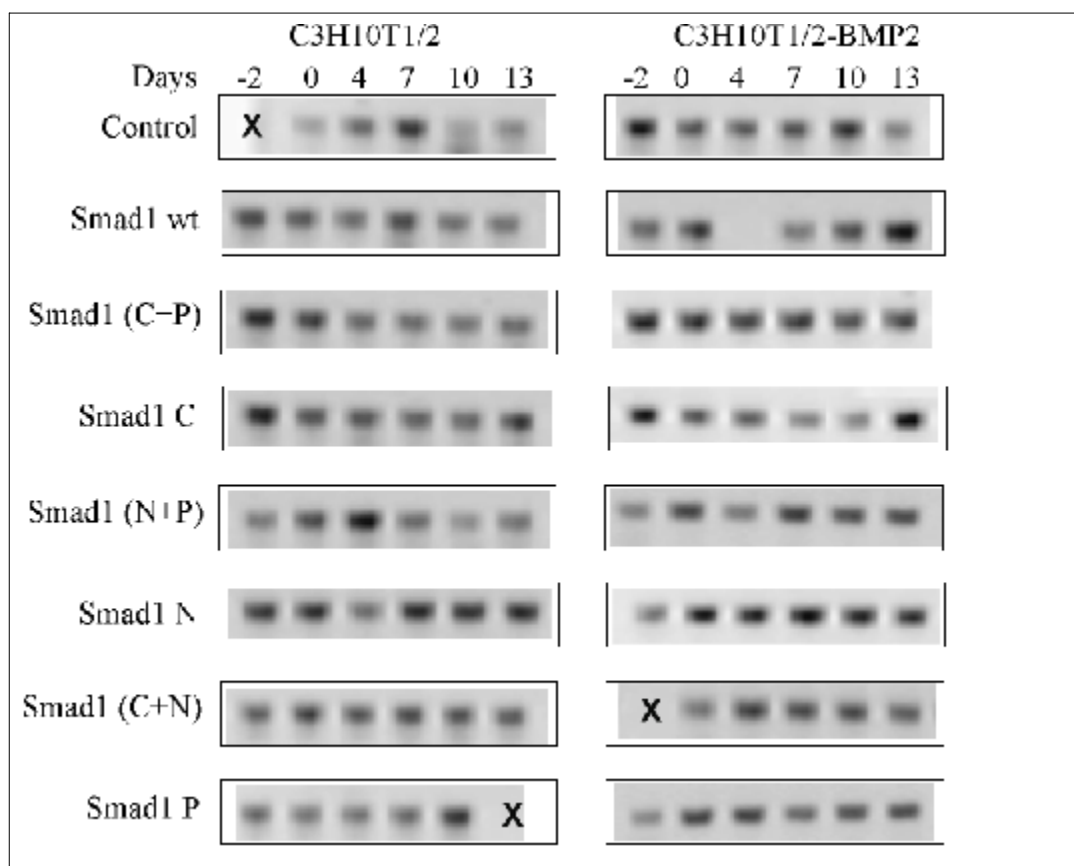


**Fig. 2.33:** RT-PCR analyses of the dose-dependent influence of Smad1 P domain on expression of the *PEX* gene in C3H10T1/2-Smad1 C cells (after equalization of cDNAs). 1 µl cDNA stock solution, 31 cycles at 52.6°C.

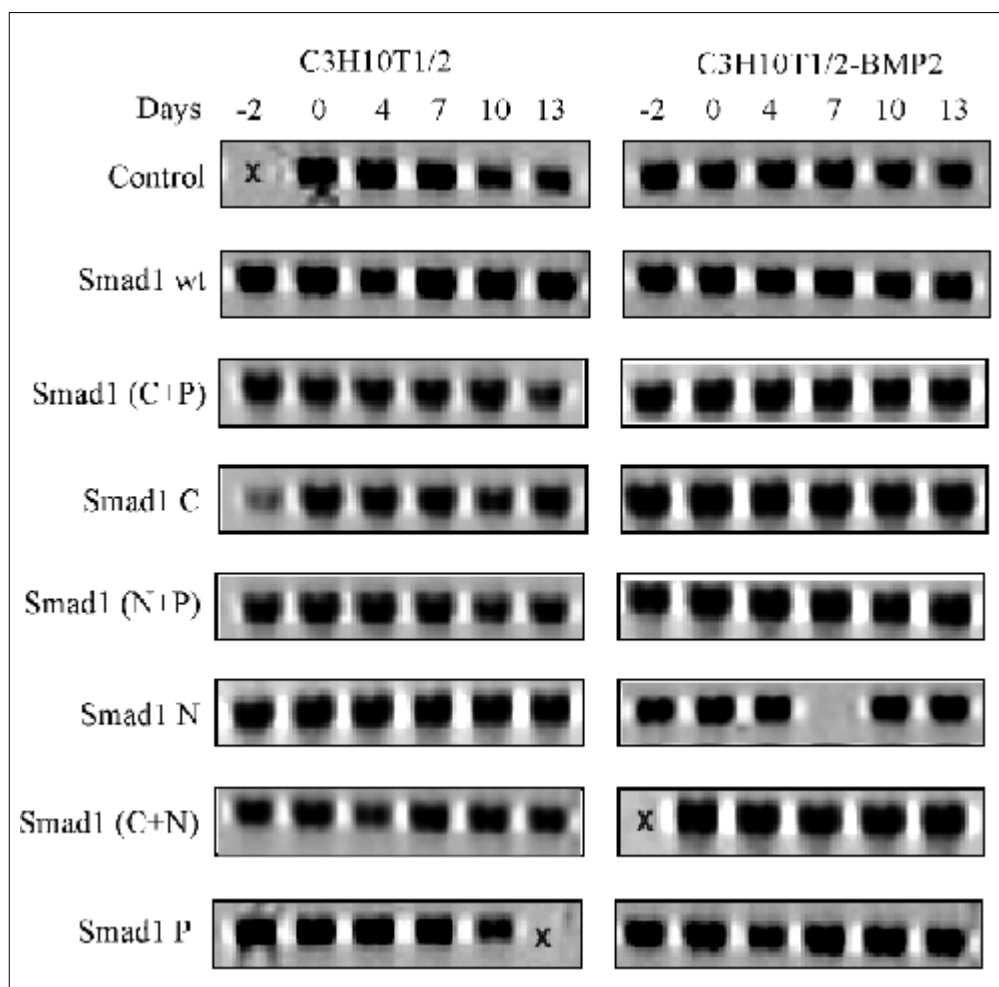
Therefore, it is likely that the Smad1 P domain exerts biological activities in regard to TGFβ or BMP-mediated signaling.

## 2.7 Endogenous expression of Smad-family members in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells

As described in the introduction, Smad1 is a BMP signal mediator, Smad2 and Smad3 are TGF- $\beta$ /Activin/Vg1 signal mediators, and Smad4 is a co-Smad which is needed in both signal transduction pathways (reviewed by Massagué et al. 1997a). Smad6 and Smad7 are anti-Smads, that serve to inhibit specific BMP and TGF- $\beta$  induced Smad signaling pathways (reviewed by Kretzschmar and Massagué 1998). To determine whether or not recombinant expression of Smad1 or its domains influence the endogenous transcriptional levels of Smad1, Smad2, Smad4 and Smad7, the C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells recombinantly expressing Smad1 and its domains were investigated through a set of primers specific for the murine genes (Appendix1: RT-Smad1, RT-Smad2, RT-Smad3, RT-Smad4 and RT-Smad7) by RT-PCR.



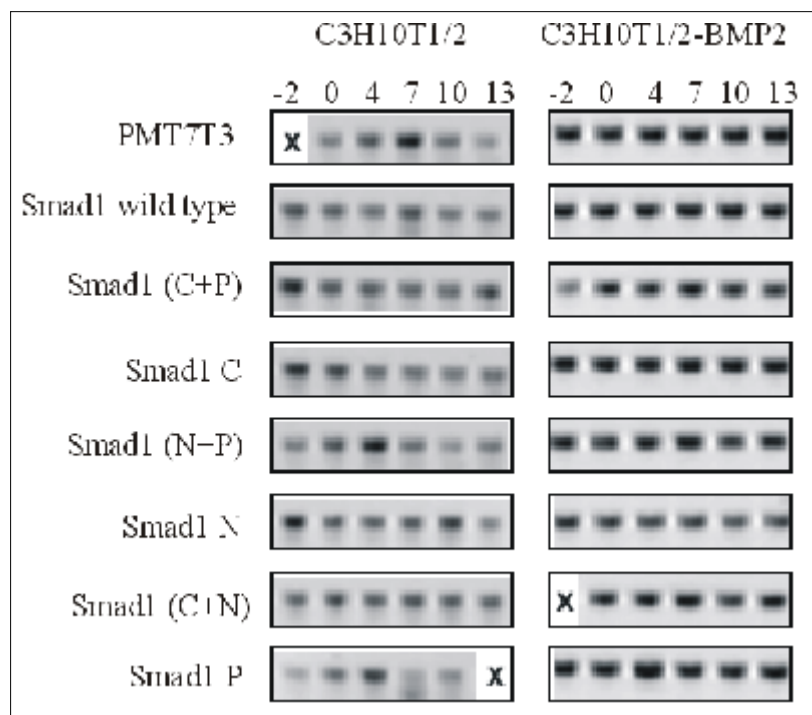
**Fig. 2.34:** RT-PCR analyses of the endogenous expression of Smad1 gene in C3H10T1/2 and C3H10T1/2<sup>BMP2</sup> cells recombiantly expressing Smad1 or its domains (after equalization of cDNAs). 2 µl 1:10 cDNA stock solution, 30 cycles at 59°C. x = RNA was lost during isolation.



**Fig. 2.35:** RT-PCR analyses of the endogenous expression of Smad2 gene in C3H10T1/2 and C3H10T1/2<sup>BMP2</sup> cells recombiantly expressing Smad1 or its domains (after equalization of cDNAs). 1 µl 1:10 cDNA stock solution, 30 cycles at 55°C. x = RNA was lost during isolation.

The C3H10T1/2 and C3H10T1/2<sup>BMP2</sup> cells recombiantly expressing Smad1 or Smad1 domains showed similar expression levels for endogenous Smad1 as the control cell lines (Fig. 2.34). Expression of Smad2 endogene in all the above cell lines behaved accordingly (Fig.

2.35). These results suggested that the endogenous expression of Smad1 and Smad2 was not affected by recombinant Smad1 or its domains.



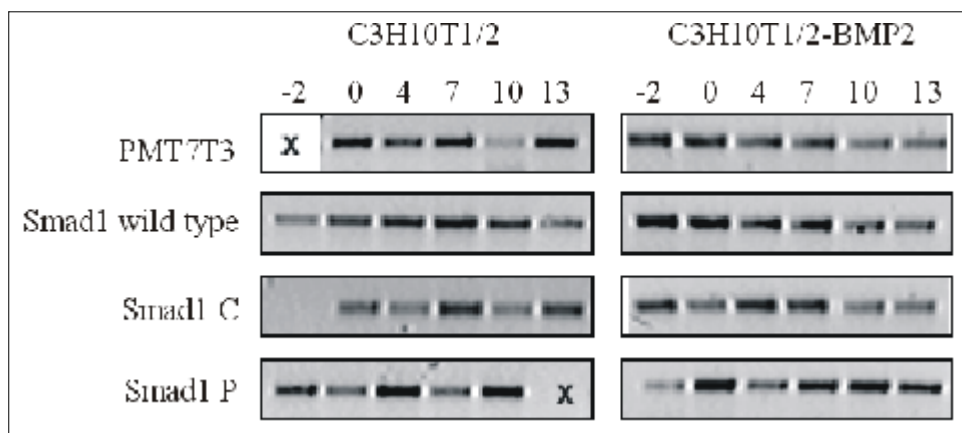
**Fig. 2.36:** RT-PCR analyses of the endogenous expression of Smad4 in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells recombinantly expressing Smad1 or Smad1 domains (after equalization of cDNAs). 1 µl 1:10 cDNA stock solution, 30 cycles at 60°C. x = RNA was lost during isolation.

As shown in Fig. 2.36, the recombinant Smad1 or Smad1 domains also did not change the endogenous expression of Smad4 in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells. However, cells with a BMP2 background showed a higher Smad4 mRNA level which was neither mimicked by the Smad1 C domain nor was it inhibited by the Smad1 P domain.

The endogenous expression of anti-Smads was also investigated. Smad6 could not be amplified by RT-PCR with the specific primers (Appendix RT-Smad6) in all the cell lines (data not shown). Smad7 was expressed in all the monitored cells at roughly the same level (Fig 2.37). The recombinant expression of Smad1 wt, Smad1 C domain and P domain did not



affect the expression level of endogenous Smad7 in C3H10T1/2 cells and C3H10T1/2<sub>BMP2</sub> cells.



**Fig. 2.37:** RT-PCR analyses of the endogenous expression of Smad7 in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells expressing Smad1 wt, Smad1 C domain and P domain (after equalization of cDNAs). 1 µl cDNA stock solution, 30 cycles at 60°C. x = RNA was lost during isolation.

Taken together, the expression of the murine endogenous Smad1-, Smad2-, Smad4- and Smad7-gene was not regulated in C3H10T1/2 cells by BMP2 or recombinant Smad1. These results imply that the endogenous Smad1-, Smad2-, Smad4- and Smad7-gene may not contribute to the BMP2- or Smad1- dependent osteogenic development in C3H10T1/2 cells.

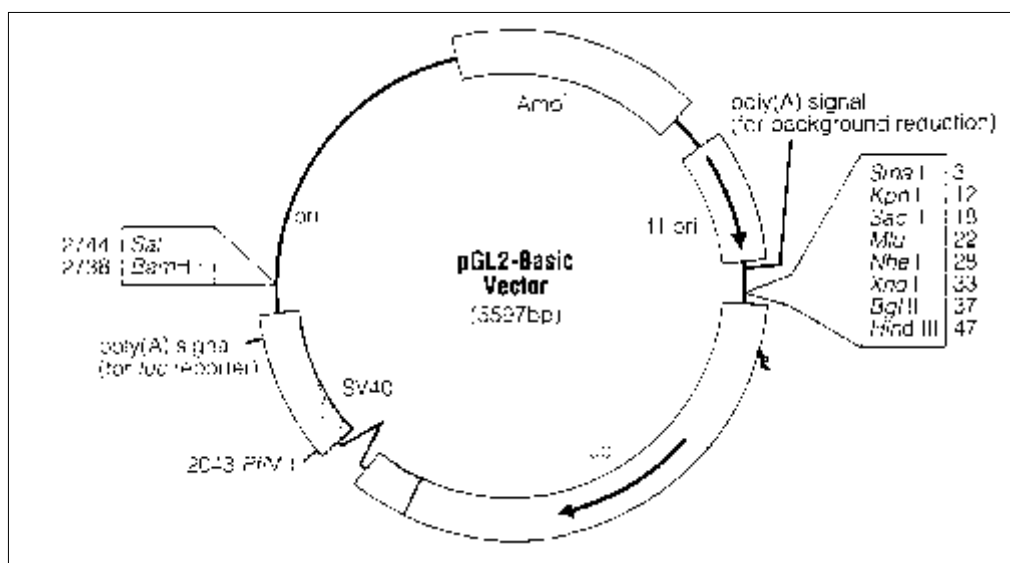
## 2.8 Transcriptional response assays

The morphological and genetic characterization of the C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells recombinantly expressing Smad1 and its domains showed that wild type Smad1 and the Smad1 C domain are able to induce C3H10T1/2 cells or to stimulate C3H10T1/2<sub>BMP2</sub> cells to differentiate into osteoblasts. The Smad1 N domain and Smad1 P domain function as negative regulators of this process. BMP-responsive promoters were used to investigate as to whether or not this negative action on osteogenesis is reflected on the level of transcription. Following promoters: 3TP-Lux, Msx-1, PTH/PTHrP receptor, JunB and a luciferase reporter were used to investigate this possibility.

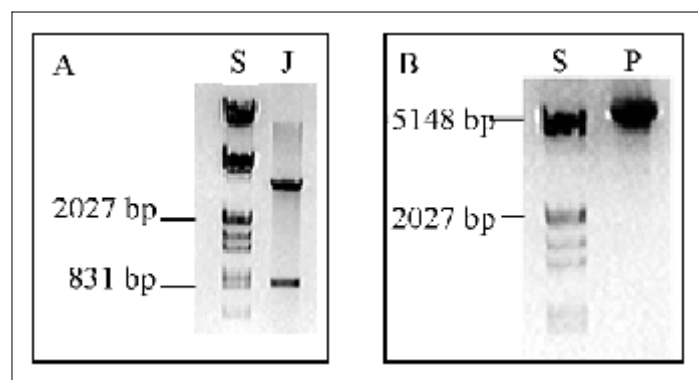
A vector harboring 3TP-Lux promoter was stably transfected into C243 cells; in addition, three vectors with the Msx-1 promoter constructs (from R. Raghow, USA), the PTH/PTHrP receptor promoter constructs (from M. Karperien, Netherlands), the JunB promoter construct (from P. Angel, Heidelberg) (-816/+28) which was subcloned into the pGL2-basic luciferase vector, all these vectors were also routinely used for transient assays. The results of luciferase assays were normalized with  $\beta$ -galactosidase activity, therefore transient transfections were done in the presence of a  $\beta$ -galactosidase reporter under the control of a SV40 early promoter.

### 2.8.1 Construction of JunB (-816/+28) promoter-luciferase expression vector

Originally the JunB promoter region -816/+388 was in a Bluescript pSK vector. To investigate the mechanism of activation of JunB promoter by BMP2, a JunB promoter-luciferase reporter was constructed containing the JunB TATA box and transcription start site, and -816 bases start site upstream sequences. The *BamH* I site at +28 and the *BamH* I site in the vector (+1927) were used for the restriction digestion. The pGL2-basic vector was also digested with *Bgl* II in order to get a compatible ends.



**Fig. 2.38:** pGL2-basic vector map.



**Fig. 2.39:** Restriction digestion of JunB promoter (-816/+388) in pSK with *BamH* I and of luciferase reporter vector pGL2-basic with *Bgl* II.

A: JunB promoter (-816/+388) in pSK digested with *BamH* I.

S = DNA standard (Lambda DNA/*EcoR* I + *Hind* III marker)

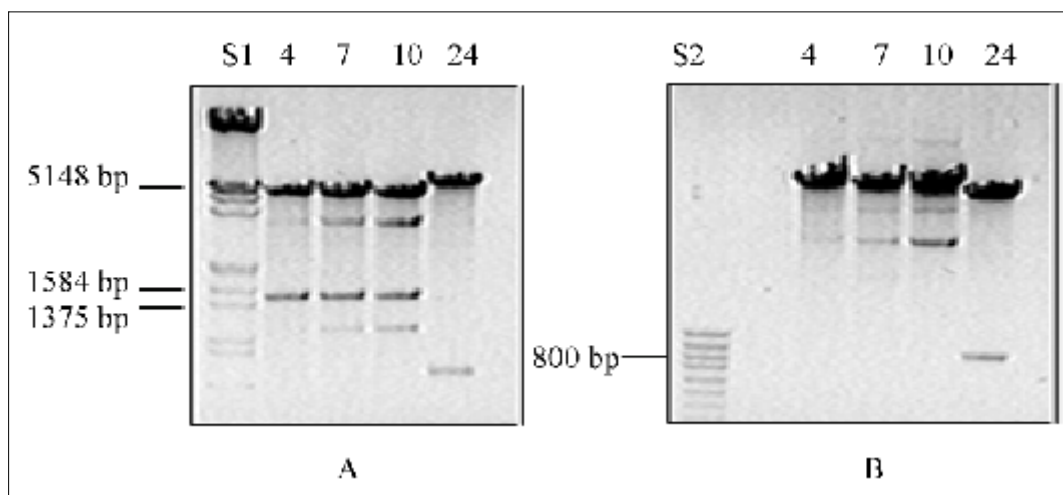
J = JunB promoter (-816/+388) in pSK

B: pGL2-basic digested with *Bgl* II.

S = DNA standard (Lambda DNA/*EcoR* I + *Hind* III marker)

P = pGL2-basic

The ligation was carried out with Ready-To-Go T4 DNA ligase kit at 16°C overnight. High voltage electroporation was performed for transformation. After minipreps of the clones, restriction digestions were performed with *EcoR* I and *Hind* III respectively in order to identify the positive clones with the right orientation of the insert (Fig. 2.40).



**Fig. 2.40:** Digestion of clone 4, 7, 10, 24 with *EcoR* I or *Hind* III.

A: Clones digested with *EcoR* I .

S1 = DNA standard (Lambda DNA/*EcoR* I + *Hind* III marker)

B: Clones digested with *Hind* III.

S2 = DNA standard (100 bp DNA ladder)

There are one *EcoR* I site and one *Hind* III site in the insert and so are there in the pGL2-basic vector. If the insert was in the right orientation, after digestion with *EcoR* I or *Hind* III there should be two fragments: about 5.0 kb/1.4 kb (for *EcoR* I digestion) and 6.4 kb/ 65bp (for *Hind* III digestion); If the insert was in the wrong orientation, there would be two fragments of about 5.8 kb/0.6 kb (for *EcoR* I) and 5.6 kb/ 0.8 kb (for *Hind* III). As shown in Fig. 2.40, the inserts in clone Nr. 4, 7 and 10 were in the right orientation, the insert in clone Nr. 24 was in the wrong orientation. The clone Nr. 4 was chosen as the JunB promoter-luciferase construct for the later experiments.

### **2.8.2 Transient transfections of the BMP-regulated promoter-luciferase constructs into eukaryotic cells**

The cells were plated at a density of 5000 cell/cm<sup>2</sup> into 12-well-plates 24 hours before transient transfection. The transfections were performed with the Dosper Liposomal Transfection Reagent kit (Chapter 5.5.6.2). For each well the transfection buffer included 1.5 µg expression vector for control or Smad1 domain DNAs, 0.5 µg promoter-luciferase construct and 0.1 µg β-galactosidase DNA. When increasing amounts of expression vectors were transfected, total DNA was kept constant by addition of control vector pMT7T3. The luciferase activity and β-galactosidase activity assays were performed two days after transient transfections.

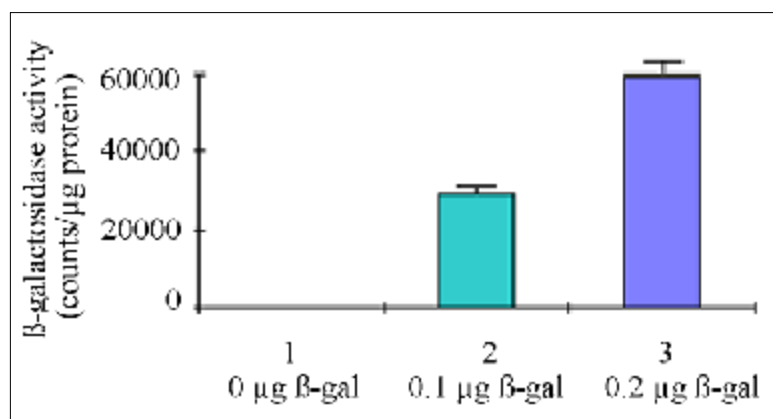
### **2.8.3 Luciferase assays of the promoter-luciferase constructs transfected cells**

The transient transfected cells were isolated with the lysis buffer in β-Galactosidase Reporter Gene Assay Kit, because the lysis buffer is fully compatible with luciferase assay procedures. The luciferase activity was measured with a luminometer. β-galactosidase activity was measured (Chapter 5.4.10.2) to determine the transfection efficiency in each assay, and the luciferase activity was standardized in regard to the β-galactosidase activity.

#### **2.8.3.1 The β-galactosidase expression vector dependent β-galactosidase activity in transient assays**

In order to use the β-galactosidase as a mean to evaluate the transfection efficiency, it is necessary to show that the amount of β-galactosidase expression vector has a proportional influence on the β-galactosidase activity. Therefore, C3H10T1/2 cells were transiently transfected with 0.1 or 0.2 µg β-galactosidase expression vector in the presence of pMT7T3 vector (to keep the total DNA amount constant in each transfection). The cell lysates were incubated at 50°C for 60 min. to reduce background counts. Then the protein concentration

was measured with the Micro BCA Protein Assay Reagent kit (Pierce). It is demonstrated in Fig. 2.41 that a relatively proportional relation exists between the amount of  $\beta$ -galactosidase expression vector and  $\beta$ -galactosidase activity. So, in this range the  $\beta$ -galactosidase activity may be regarded as a reliable criterion of transfection efficiencies.

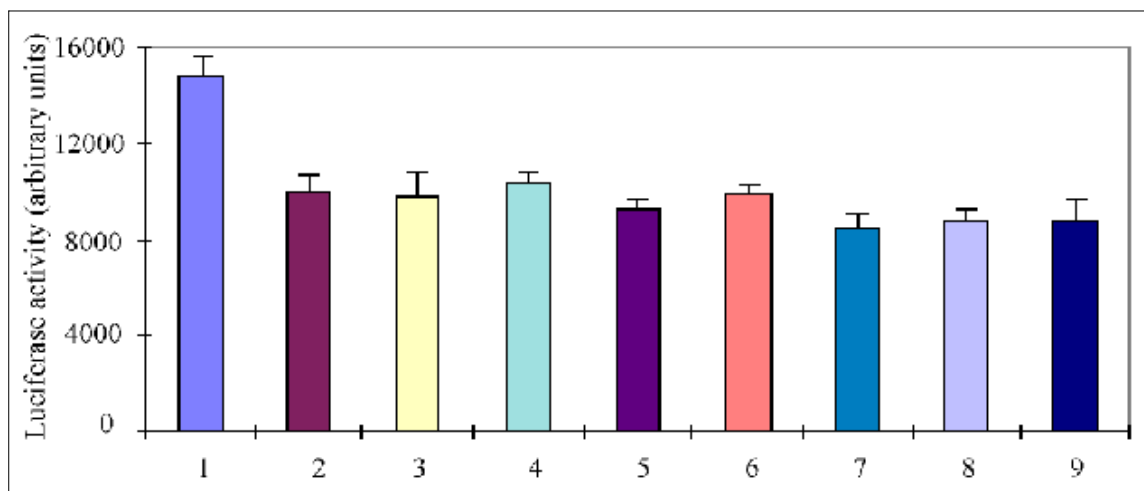


**Fig. 2.41:** The proportional relation between  $\beta$ -galactosidase vector amount and  $\beta$ -galactosidase activity. The values were determined as mean  $\pm$  S.E. (standard error) of three separate transfections.

### 2.8.3.2 Smad1 and its domains do not activate the p3TP-lux promoter-luciferase construct in murine C243 cells

The p3TP-lux luciferase reporter is a well-described and widely used artificial promoter construct which was empirically designed to have maximal responsiveness to TGF- $\beta$  (Wrana et al. 1992). p3TP-lux has a 31-nucleotide, AP1 site-containing region of the collagenase promoter, concatemerized 5' to an about 400-nucleotide region of the plasminogen activator inhibitor type I (PAI-1) promoter followed by 70 bp of the adenovirus E4 promoter (Yingling et al. 1997). Smad2, Smad3 and Smad4 have been shown to play some roles in potentiating TGF- $\beta$ /activin stimulation of transcription of the p3TP-lux construct (Chen et al. 1996; Lagna et al. 1996). To test whether *Xenopus* Smad1 has a stimulatory effect on p3TP-lux reporter activity, the murine C243 cells with the stable incorporation of 3TP-Lux vector

(C243-p3TP-lux) were transiently transfected with Smad1 domains and luciferase activity was then measured.



**Fig. 2.42:** The influence of recombinant Smad1 and Smad1 domains on p3TP-lux luciferase activity (after normalization with  $\beta$ -galactosidase activity). The values were determined as mean  $\pm$  S.E. (standard error) of three separate transfections.

1. C243-p3TP-lux cells
2. C243-p3TP-lux/control vector
3. C243-p3TP-lux/wild type Smad1
4. C243-p3TP-lux/Smad1 C domain
5. C243-p3TP-lux/Smad1 (C+P) domain
6. C243-p3TP-lux/Smad1 N domain
7. C243-p3TP-lux/Smad1 (N+P) domain
8. C243-p3TP-lux/Smad1 P domain
9. C243-p3TP-lux/Smad1 (C+N) domain

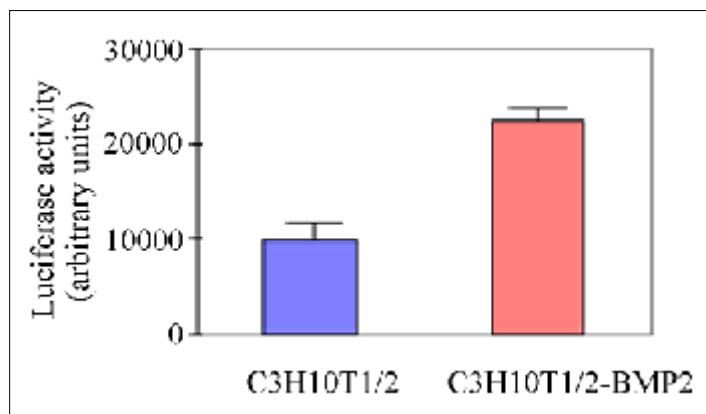
Fig. 2.42 showed that recombinant Smad1 and its domains have neither stimulated nor inhibited the luciferase activity of the p3TP-lux in C243 cells. This may be explained by the fact that the C243 cell line is not a TGF- $\beta$  or BMP2 responsive cell line, therefore the BMP-responsive procedures were then characterized in the mesenchymal progenitors C3H10T1/2 cells.

### 2.8.3.3 The influence of Smad1 domains on the JunB promoter (-816/+28)-luciferase activity

The product of the JunB gene is a member of the AP-1 family of transcription factors that activate transcription by binding to TPA responsive elements (TREs) within the promoter of the target genes (Angel et al. 1987). AP-1 components are immediately early gene products whose expression is rapidly induced by a variety of extracellular stimuli and are encoded by the Fos and Jun families of genes that have been shown to be involved in growth control and differentiation (Angel and Karin 1991). JunB differs in biological properties from its homologs and appears to be a negative regulator of AP-1 function (Chiu et al 1989). The action of JunB as a negative regulator of TRE response elements is consistent with its induction by negative regulators of cell growth including TGF- $\beta$  as well as activin and BMP2/4 (de Groot and Kruijer 1991; Li et al. 1990; Hashimoto et al. 1993; Chalaux et al. 1998). Developmental studies performed *in vitro* and *in vivo* implicate that the Fos and Jun family of transcription factors play a role in regulation of bone tissue formation (Johnson et al. 1992; Reimold et al. 1996). From all AP-1 transcription factors studied, only JunB is highly induced at early times after BMP2 or TGF- $\beta$  addition (Chalaux et al. 1998). The JunB is not affected by the protein synthesis inhibitor cycloheximide suggesting the transacting factors required for transcription pre-exist in the cell, and the Smad family may be the direct mediators of this JunB transcriptional activation (Chalaux et al. 1998).

The JunB promoter (-816/+28)-luciferase construct was transiently transfected into C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells to test whether the BMP2 background leads to enhancing transcriptional activity directed by the JunB promoter in C3H10T1/2 cells.

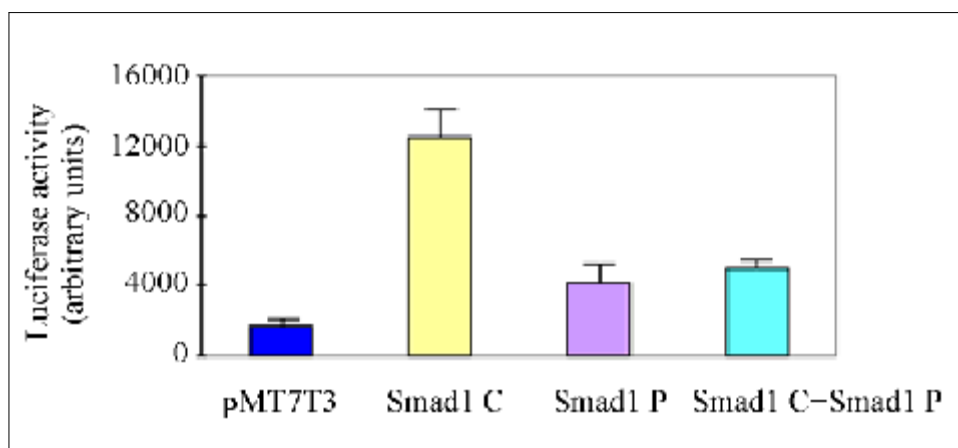




**Fig. 2.43:** The luciferase activity mediated by the JunB promoter (-816/+28) construct in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells (after normalization with  $\beta$ -galactosidase activity). The values were determined as mean  $\pm$  S.E. (standard error) of three separate transfections.

The JunB promoter-luciferase activity in a BMP2 background is about 2-fold enhanced in comparison with in control C3H10T1/2 cells (Fig. 2.43). Because NIH3T3 cells exhibit similar results but by far better transfection characteristics than C3H10T1/2 cells, more detailed analyses were performed in these cells. Whether the BMP signal mediator Smad1 has an effect on the JunB promoter activity was then investigated. In this experiments NIH3T3 cells were transfected with 1.6  $\mu$ g pMT7T3; 0.8  $\mu$ g expression vector for Smad1 C domain and 0.8  $\mu$ g pMT7T3; 0.8  $\mu$ g expression vector for Smad1 P domain and 0.8  $\mu$ g pMT7T3; 0.8  $\mu$ g expression vector for Smad1 C domain and 0.8  $\mu$ g expression vector for Smad1 P domain.

The luciferase activity assay indicated that expression of the Smad1 C domain leads to enhanced JunB promoter activity about 7-fold. The cells transfected with Smad1 P domain also increased the promoter activity 2-fold. The JunB promoter dependent luciferase activity in NIH3T3 cells cotransfected with Smad1 C domain and Smad1 P domain was higher than the transfection of the P domain alone but significantly lower than C domain transfected cells (Fig. 2.44) consistent with the earlier finding that the Smad1 P domain represses the Smad1 C domain-dependent stimulation of transcription.



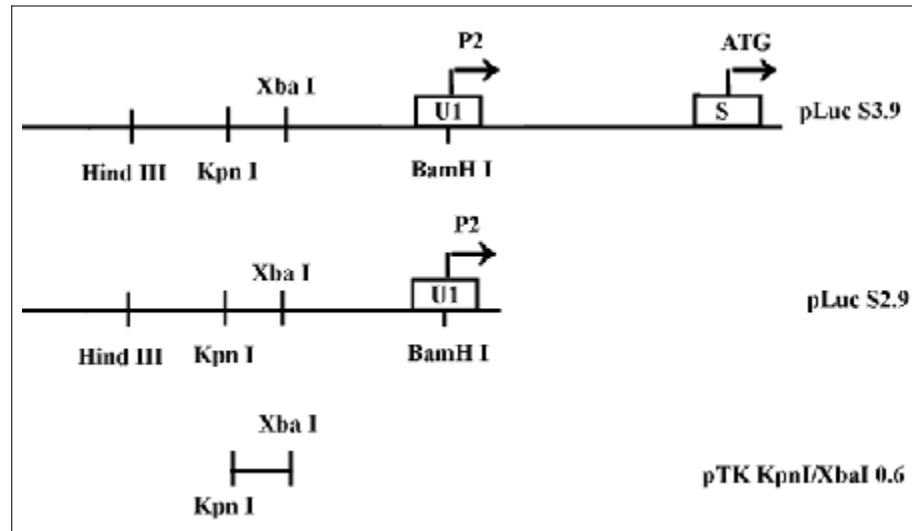
**Fig. 2.44:** The influence of Smad1 C domain, P domain, and C domain + P domain on JunB promoter (-816/+34)-luciferase activity (after normalization with  $\beta$ -galactosidase activity). The values were determined as mean  $\pm$  S.E. (standard error) of three separate transfections.

#### 2.8.3.4 The influence of Smad1 domains on the PTH/PTHrP receptor promoter-luciferase constructs activity

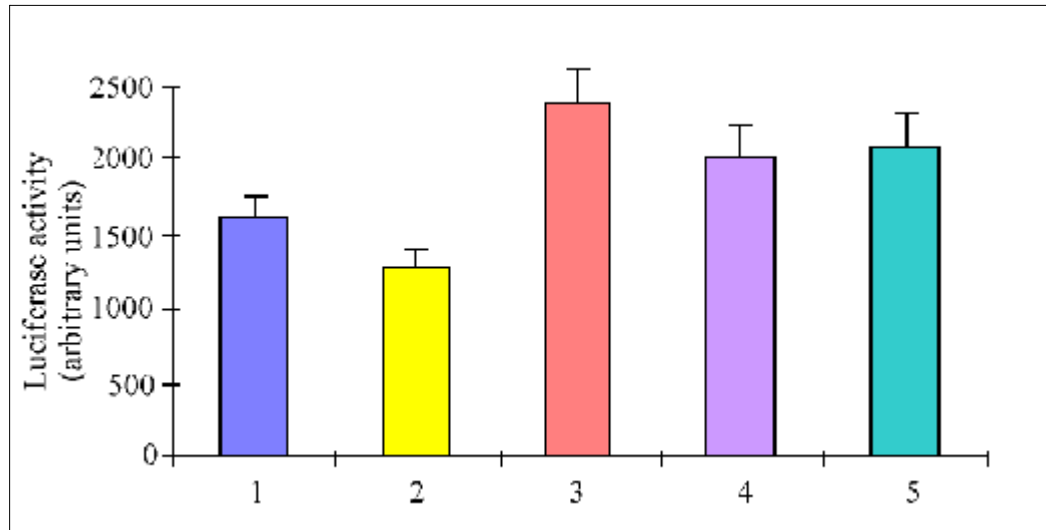
The PTH/PTHrP receptor gene expression is obviously regulated by Smad1 wild type and Smad1 C domain construct, while the Smad1 P domain abolished the PTH/PTHrP receptor expression in both C3H10T1/2<sub>BMP2</sub> cells and C3H10T1/2-Smad1 C cells (Chapter 2.3.2). So it is likely that Smad1 might induce transcriptional activation of PTH/PTHrP receptor gene.

In this regard the PTH/PTHrP receptor promoter-luciferase constructs were kindly supplied by M. Karperien, Leiden, Netherlands. Three of the PTH/PTHrP receptor promoter-luciferase constructs were tested in our system. pLuc U1 2.9: this construct contains 2.9 kb upstream sequences (*Hind* III/*Bam* H I) of the downstream P2 promoter; pLuc S 3.9: this construct contains a downstream intron between exon U1 and S, and is otherwise identical to pLuc U1

2.9; pTK 0.6 kb: the 0.6 kb promoter fragment (KpnI/XbaI) was cloned in front of the heterologous minimalTK-promoter in pTK Luc vector (Fig. 2.45).

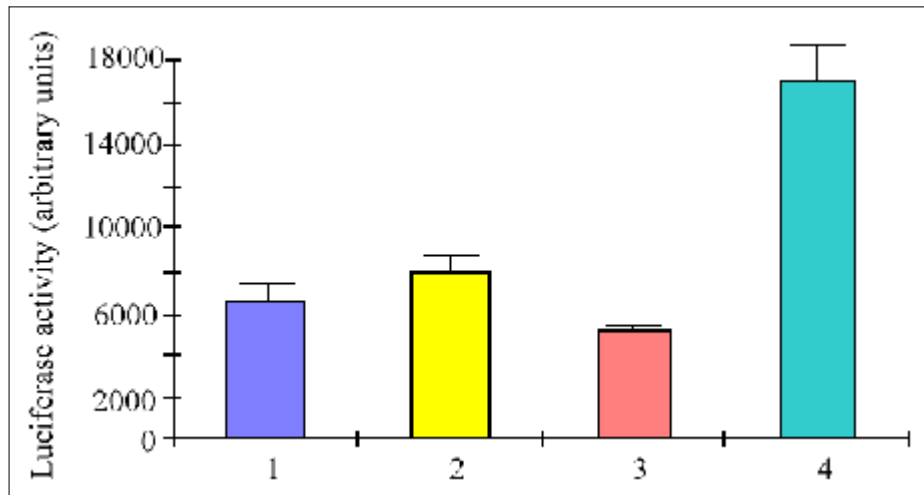


**Fig. 2.45:** Diagram of the PTH/PTHrP receptor promoter containing luciferase reporters.



**Fig. 2.46:** The influence of Smad1 domains on luciferase activity directed by the PTH/PTHrP receptor promoter (pLuc U1 2.9) (after normalization with  $\beta$ -galactosidase activity) in NIH3T3 cells. The values were determined as mean  $\pm$  S.E. (standard error) of three separate transfections.

- 1: NIH3T3/pMT7T3
- 2: NIH3T3/Smad1 C domain
- 3: NIH3T3/Smad1 wild type
- 4: NIH3T3/Smad1 P domain
- 5: NIH3T3/Smad1 N domain

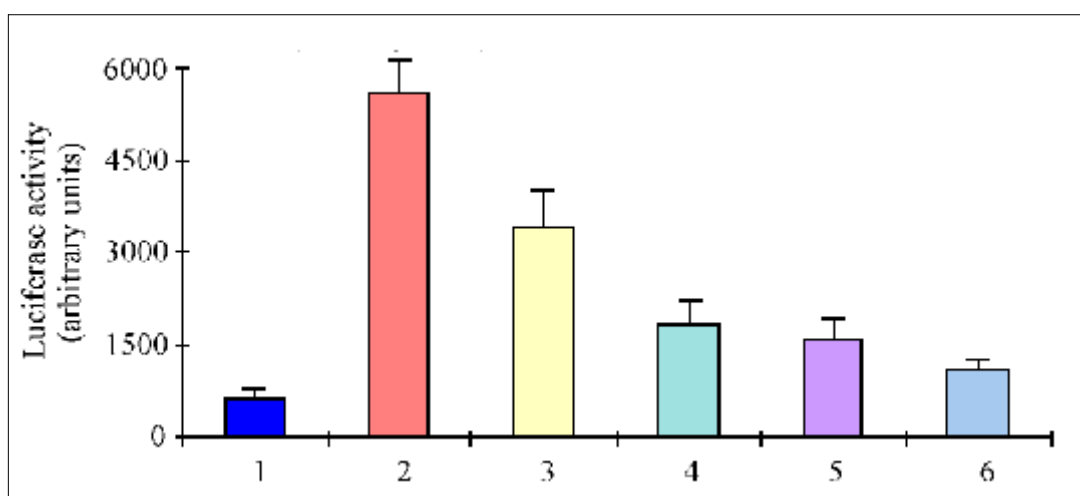


**Fig. 2.47:** The influence of Smad1 domains on luciferase activity directed by the PTH/PTHrP receptor promoter (pLuc S 3.9) (after normalization with  $\beta$ -galactosidase activity) in NIH3T3 cells. The values were determined as mean  $\pm$  S.E. (standard error) of three separate transfections.

- 1: NIH3T3/control vector
- 2: NIH3T3/Smad1 C domain
- 3: NIH3T3/Smad1 wild type
- 4: NIH3T3/Smad1 P domain

The luciferase activity of Smad1 domains transiently transfected cells (Fig. 2.46, 2.47) indicated that the PTH/PTHrP receptor promoter construct pLuc U1 2.9 and 3.9 activity are not influenced by Smad1 wild type and its domains. Consistent with this result, we learned after this experiment that sequences about 7000 bp upstream of U1 are necessary for regulated activities in rat osteosarcoma ROS17/2.8 cells (M. Karperien, personal communication).

While the luciferase activities of PTH/PTHrP receptor promoter pTK 0.6 were significantly induced about 10-fold and 6-fold in NIH3T3 cells transfected with wild type Smad1 and Smad1 C domain (Fig. 2.48). Smad1 N domain and Smad1 P domain have also stimulated the promoter activity. The promoter activity of cells cotransfected with Smad1 P domain and Smad1 C domain was significantly lower than the cells transfected with Smad1 C domain alone. This implies that the 0.6 kb promoter fragment is BMP- or Smad1-responsive element, whose activity is normally silenced by the other regions of the PTH/PTHrP receptor promoter.

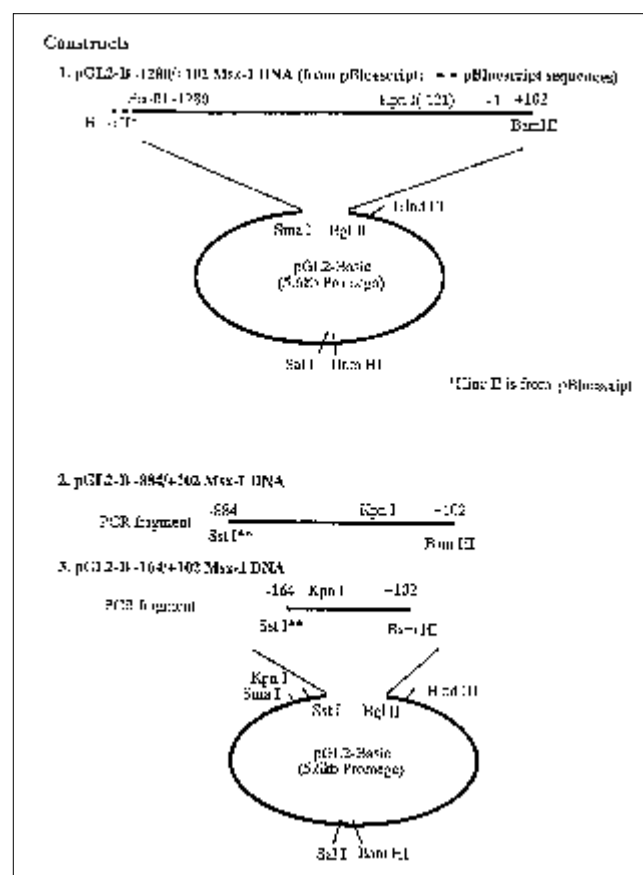


**Fig. 2.48:** The influence of Smad1 and its domains on PTH/PTHrP receptor promoter pTK 0.6 directed luciferase activity (after normalization with  $\beta$ -galactosidase activity) in NIH3T3 cells. The values were determined as mean  $\pm$  S.E. (standard error) of three separate transfections.

- 1: NIH3T3/pMT7T3
- 2: NIH3T3/Smad1 wt
- 3: NIH3T3/Smad1 C domain
- 4: NIH3T3/Smad1 N domain
- 5: NIH3T3/Smad1 P domain
- 6: NIH3T3/Smad1 C domain + Smad1 P domain

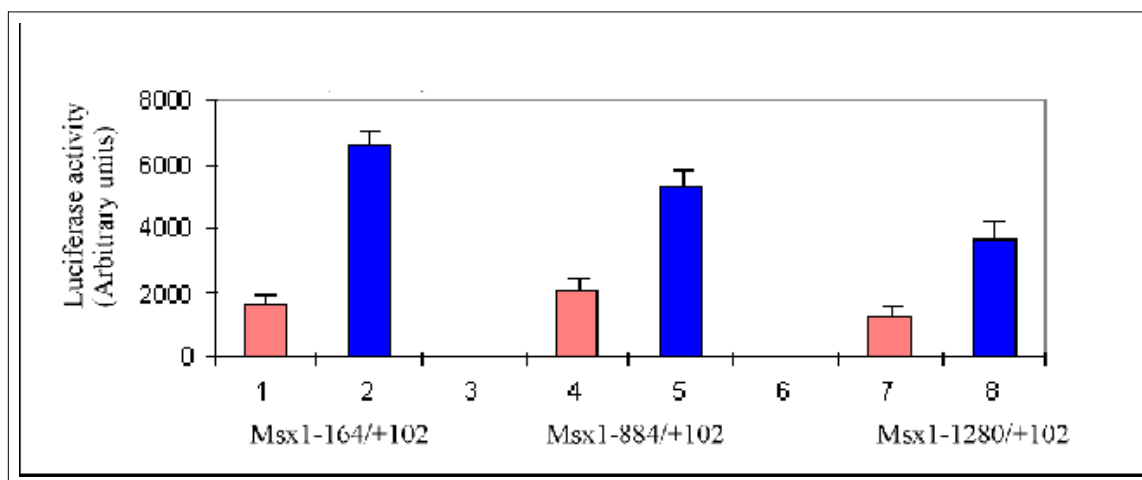
### 2.8.3.5 The influence of Smad1 domains on the Msx-1 promoter-luciferase constructs activity

Msx genes are vertebrate homologues of *Drosophila msh* (muscle segment homeobox) gene and it has been reported that they are BMP responsible genes (reviewed by Davidson 1995). Direct evidence has been found that addition of BMP2 or BMP4 can induce the expression of both Msx-1 and Msx-2 in tooth mesenchymal explants (Vainio et al. 1993). The timing of expression and embryonic distribution of Msx1 parallel that described for BMPs. Moreover, it was demonstrated that Msx1 can mimic the activities of BMPs: overexpression of Msx1 in early *Xenopus* embryos leads to ventralization (Suzuki et al. 1997). The effect of Smad1 on the downstream factors of BMP was investigated in Msx1 promoter-luciferase assay level. We obtained Msx1 luciferase reporter constructs from R. Raghov, Memphis.



**Fig. 2.49:** Maps of Msx1 promoter fragments in pGL2-basic vector.

Three different length Msx1 promoter fragments (-1280/+102; -884/+102; and -164/+102) have been cloned into pGL2-basic vector (Fig. 2.49). They were transiently transfected into C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells to test whether or not BMP2 has an effect on Msx1 promoter-luciferase activity.



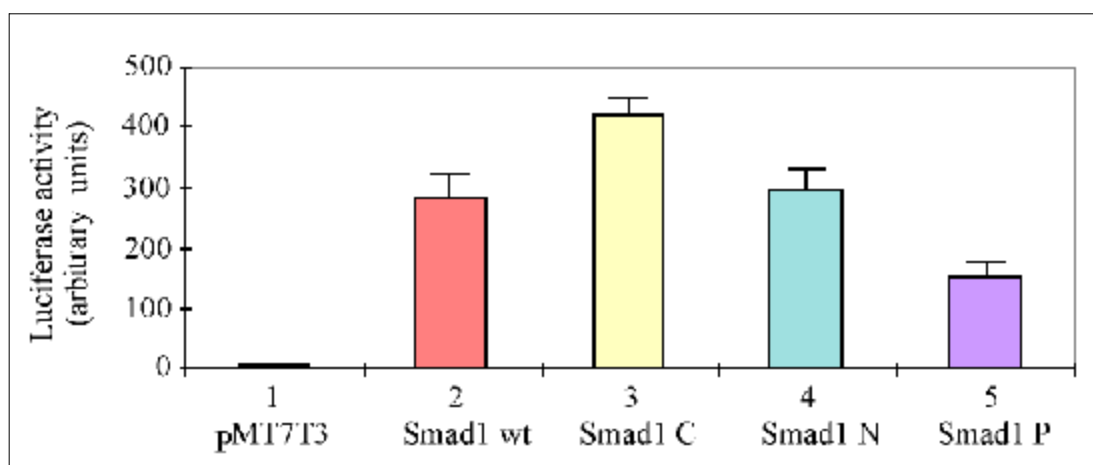
**Fig. 2.50:** The activity of three Msx1 promoter-luciferase constructs in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells (after normalization to  $\beta$ -galactosidase activity). The values were determined as mean  $\pm$  S.E. (standard error) of three separate transfections.

- 1, 4, 7: C3H10T1/2 cells transfected with three Msx1 promoter constructs
- 2, 5, 8: C3H10T1/2<sub>BMP2</sub> cells transfected with three Msx1 promoter constructs

As shown in Fig. 2.50, all three Msx1 promoter-luciferase constructs were all stimulated by BMP2, though at different efficiency. In C3H10T1/2<sub>BMP2</sub> cells the Msx1-164/+102, the Msx1-884/+102, and the Msx1-1280/+102 promoter-luciferase activity are respectively 3-fold, 1.6-fold and 1.9-fold higher than in C3H10T1/2 cells. The optimal stimulation of BMP2 on luciferase activity was observed in Msx1-164/+102 promoter region, so additional experiments were performed with this promoter-luciferase construct .

BMP2 seems to exert a 3-fold stimulation on the Msx1-164/+102 promoter-luciferase activity. The effect of the BMP2-signal mediator Smad1 and its domains on the Msx1-164/+102

promoter-luciferase was then tested in cotransfection experiments in the parental C3H10T1/2 cells. In this experiments control vector pMT7T3, wild type Smad1, Smad1 C domain, Smad1 N domain and Smad1 P domain in the presence of Msx1-164/+102 promoter-luciferase construct and  $\beta$ -galactosidase expression vector were transiently transfected into C3H10T1/2 cells. The results of luciferase activity was normalized for  $\beta$ -galactosidase activity.



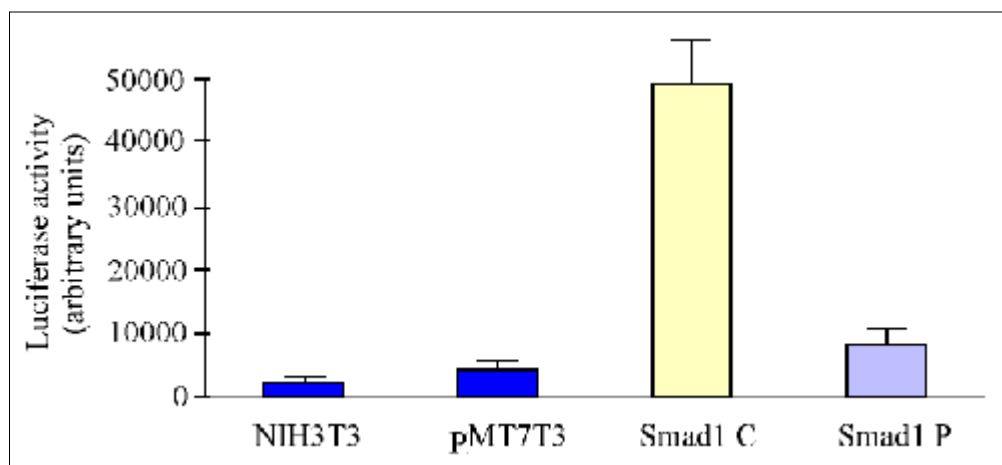
**Fig. 2.51:** The influence of recombinant Smad1 wild type, C domain, N domain and P domain on Msx1-164/+102 promoter-luciferase construct activity in C3H10T1/2 cells (after normalization with  $\beta$ -galactosidase activity). The values were determined as mean  $\pm$  S.E. (standard error) of three separate transfections.

1, 2, 3, 4, 5: C3H10T1/2 cells were transfected with expression vector pMT7T3, wild type Smad1, C domain, N domain and P domain.

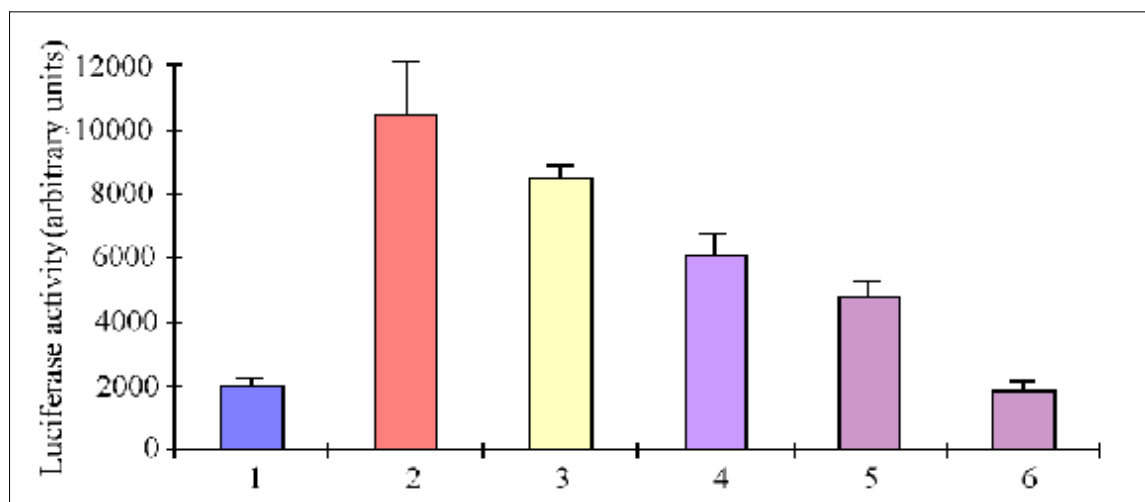
The recombinant Smad1 and Smad1 C domain, N domain and even the P domain have stimulated the luciferase activity under the control of Msx1-164/+102 promoter-luciferase constructs (Fig. 2.51). The cells transfected with Smad1 C domain construct showed the highest activity. Interestingly, also the Smad1 P domain stimulates luciferase activity although lower in comparison with the other constructs.



The stimulation of Smad1 C domain on Msx1-164/+102 promoter-luciferase activity was also confirmed in NIH3T3 cells (Fig. 2.52). The NIH3T3 cells have better efficiencies in transient transfections and showed a similar effect as C3H10T1/2 cells after being transfected with Smad1 domains. Therefore, the later transient transfections were normally carried out in NIH3T3 cells. The Smad1 C domain and P domain have both stimulated the luciferase activity, Smad1 C domain showed about 10-fold stimulation, and Smad1 P domain has only about 1.5-fold stimulation compared with the control vector. Smad1 C domain transfected cells have about 6-fold higher luciferase activity in comparison with Smad1 P domain transfected cells.



**Fig. 2.52:** The influence of Smad1 C domain and P domain on Msx1-164/+102 promoter activity in NIH3T3 cells (after normalization with  $\beta$ -galactosidase). The values were determined as mean  $\pm$  S.E. (standard error) of three separate transfections.



**Fig. 2.53:** The influence of Smad1 domain constructs on Msx1-164/+102 promoter activity in NIH3T3 cells (after normalization with  $\beta$ -galactosidase). The values were determined as mean  $\pm$  S.E. (standard error) of three separate transfections.

- 1: NIH3T3/control vector
- 2: NIH3T3/wild type Smad1
- 3: NIH3T3/Smad1 C domain
- 4: NIH3T3/Smad1 P domain
- 5: NIH3T3/Smad1 C + 1/2 Smad1 P domain
- 6: NIH3T3/Smad1 C + 1 Smad1 P domain

As shown in Fig. 2.53 the interference between Smad1 C domain and Smad1 P domain was investigated in NIH3T3 cells. 2.2  $\mu$ g total DNA was used for each transfection, of which 0.5  $\mu$ g was Msx1-164/+102 promoter construct, 0.1  $\mu$ g was  $\beta$ -galactosidase vector, the remaining 1.6  $\mu$ g were control vector or Smad1 domains in an expression vector. Panel 2, 3 and 4 represent the luciferase activity of NIH3T3 cells transfected with 0.8  $\mu$ g expression vector for Smad1 wild type, Smad1 C domain, Smad1 P domain, Smad1 (C+N) domain, respectively, and 0.8  $\mu$ g pMT7T3 vector DNA. Panel 5 shows the luciferase activity of cells transfected with 0.8  $\mu$ g expression vector for Smad1 C domain + 0.4  $\mu$ g expression vector for Smad1 P domain + 0.4  $\mu$ g pMT7T3 vector DNA. Panel 6 shows the luciferase activity of cells

transfected with 0.8 µg expression vector for Smad1 C domain + 0.8 µg expression vector for Smad1 P domain.

Smad1 wild type, Smad1 C domain, and Smad1 P domain showed an about 4-fold, 3-fold, and 2-fold induction in comparison with control vector. Panel5 and 6 documented the dose-dependent interference of Smad1 P domain with Smad1 C domain dependent biological activities.

In total, the experiments document that one can substantiated with BMP-responsive promoters the histological and genetic analyses of Smad signaling-mediators in mesenchymal progenitors C3H10T1/2 cells.

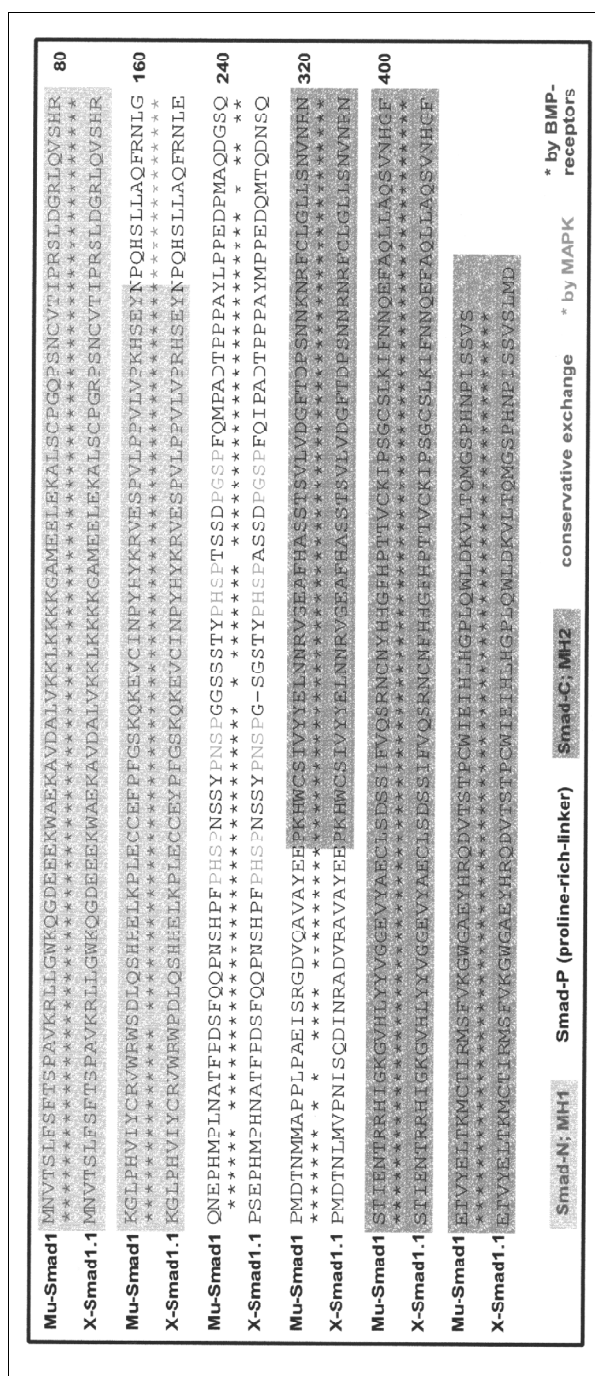
### **3 Discussion**

#### **3.1 The effects of overexpression of *Xenopus* Smad1 on osteogenic, chondrogenic and adipogenic development in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells**

C3H10T1/2 cells represent a relatively early stage of mesenchymal cell determination with the ability to differentiate into osteoblasts, adipocytes and chondrocytes. Their multipotential nature and their responsiveness toward TGF- $\beta$  and BMP treatment make this cell line a useful model system to explore the involvement of factors in various mesenchymal differentiation processes. A series of studies revealed that BMP2 has the ability to stimulate osteogenesis in predetermined osteogenic cell lines and to induce osteogenesis in undetermined mesenchymal progenitor cell line (Chen et al. 1991; Tokuwa et al. 1991; Thies et al. 1992). In C3H10T1/2 cells, BMP initiates development into the osteogenic, chondrogenic and adipogenic lineage (Ahrens et al. 1993; Wang et al 1993).

Smad1's function, especially the function of its three domains, was investigated in BMP2-dependent osteo/chondrogenic mesenchymal differentiation system. The *Xenopus* recombinant Smad1 and its domains were stably transfected into the murine, mesenchymal progenitor cell line C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells (Chapter 2.1.2 to 2.1.4). The *Xenopus* Smad1 is highly identical with murine Smad1, even the P domains shares a 89.1% identity with the corresponding sequence in murine Smad1 (Fig. 3.1).

The osteogenic differentiation potential of C3H10T1/2 cells was assessed by alkaline phosphatase staining (Fig. 2.7; 2.8; 2.9; 2.10) and genetic analyses of marker genes typical or specific for osteogenesis, including: osteocalcin (Fig. 2.13), PTH/PTHrP receptor (Fig. 2.14), collagen I (Fig. 2. 15), *CBFA1* (Fig. 2.16), osteopontin (Fig. 2.17), and osteonectin (Fig. 2.18); the chondrogenic differentiation potential of C3H10T1/2 cells was assessed by Alcian Blue staining (Fig. 2.11) and genetic analyses of marker genes typical or specific for chondrogenesis: collagen II (Fig. 2.19) and FGFR-3 (Fig. 2.20); The adipogenic potential of C3H10T1/2 cells was identified morphologically (Fig. 2.4; 2.5, 2.6) and by the adipoQ gene expression (Fig. 2. 21), which is specific for adipogenesis (Chapter 2.2 and 2.3).



**Fig. 3.1:** Protein sequence alignment between *Xenopus* Smad1 and murine Smad1.

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In this investigation we show that the BMP-2 dependent signaling molecule Smad1 or its biological active domain (C domain) induced the alkaline phosphatase activity (Fig. 2.9) and the expression of osteogenesis marker genes such as osteocalcin (Fig. 2.13), PTH/PTHrP receptor (Fig. 2.14) and collagen I (Fig. 2.15) in C3H10T1/2 cells. In addition, in C3H10T1/2<sub>BMP2</sub> cells, the BMP2 dependent activation of alkaline phosphatase (Fig. 2.10), osteocalcin (Fig. 2.13), PTH/PTHrP receptor (Fig. 2.14) and collagen I gene (Fig. 2.15) was enhanced by Smad1 or its biological active domains. Therefore, on the basis of histological and genetic analyses, Smad1, as the BMP2 signaling transducer, was demonstrated to play an important role in inducing the C3H10T1/2 cells differentiation into osteogenic lineage.

Chondrocytes were identified in C3H10T1/2 cells expressing BMP2/BMP4 on the basis of Alcian Blue staining (Ahrens et al. 1993). Whether Smad1 or its domains mediated the BMP2 dependent chondrogenesis was investigated in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells. Chondrocytes were not detected in C3H10T1/2 cells expressing recombinant Smad1 or its domains by Alcian Blue staining (Fig. 2.11), which implies that the recombinant Smad1 and its domains are not sufficient to initiate the development of C3H10T1/2 cells into chondrogenic lineage. Chondrocyte-formation was observed in C3H10T1/2<sub>BMP2</sub> cells expressing Smad1 wild type, C domain or P domain, however, at a level comparable with that of control C3H10T1/2<sub>BMP2</sub> cells. Furthermore, the expression of chondrogenic marker genes, such as collagen II or FGFR-3 was not regulated by recombinant Smad1 or Smad1 domains (Chapter 2.3.7 and 2.3.8). Therefore, it can be anticipated from these results that the chondrogenic potential of C3H10T1/2<sub>BMP2</sub> cells was neither stimulated by overexpression of Smad1 biological active domains nor inhibited by overexpression of the Smad1 P domain, the negative regulator for osteogenesis. BMP2-dependent chondrogenesis appears to be mediated by other signaling molecules other than Smad1. Smad5, Smad8, or mediators of the MAP-kinase pathway, such as TAK1, or TAB1 could exert the induction of BMP2-induced chondrogenic development.

The role of BMPs in adipogenesis is less clearly defined since several studies indicate a BMP-dependent stimulation while other reports describe an inhibition of adipogenesis in differentiation systems like those established from bone marrow stroma cells (Gimble et al.

### **3 Discussion**

1995). In BMP2 expressing C3H10T1/2 cells adipocytes were observed during 15-days of cultivation (Ahrens et al. 1993). However, the expression of adipoQ in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells expressing recombinant Smad1 or its domains, combined with the erratic appearance of adipocytes in the above cell lines (Fig. 2.5 and 2.6), implied that BMP2 contributes to adipoQ expression and the number of adipocytes in C3H10T1/2 cells. However, other factors, in addition to Smad1, might play more important roles in the BMP2-dependent adipogenesis.

The effect of recombinant expression of *Xenopus* Smad1 and its domains on BMP2-dependent osteogenesis in C3H10T1/2 cells was not due to up/down-regulation of the endogenous R-Smads, Co-Smad or Anti-Smads, because overexpression of Smad1 has no obvious influence on the endogenous expression level of Smad1, Smad2, Smad4 and Smad7 (Fig. 2.34; 2.35; 2.36; 2.37). In addition, the RT-PCR analyse with Smad4 suggests that the endogenous level of Smad4 is sufficient for co-operation with the overexpressed Smad1 to carry out the nuclear functions.

## 3.2 The different roles of the Smad1 N terminal domain, proline-rich linker region and C terminal domain in osteogenic development of C3H10T1/2 cells

As described above, except *CBFA1* and the osteopontin and osteonectin genes, the osteogenetic marker genes, including alkaline phosphatase, PTH/PTHrP receptor, collagen I gene were all regulated by Smad1 and its domains in C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells (Chapter 2.2; 2.3). In addition, the three domains of Smad1 were shown to play different roles in this process (Table 3.1).

Domain Effect Gene	N C3H10T1/2	N C3H10T1/2 <sub>BMP2</sub>	P C3H10T1/2	P C3H10T1/2 <sub>BMP2</sub>	C C3H10T1/2	C C3H10T1/2 <sub>BMP2</sub>
Alkaline Phosphatase	*	↓	*	↓	↑↑	↑
Collagen I	↓	↓	↓	↓	↑	↑
PTH/PTHrP receptor	↑	↓	↓	↓	↑	*
Osteopontin	↓	↓	↑	*	*	*
Osteonectin	*	*	*	*	*	*
Osteocalcin	↑	*	↓	↓	↑	↑
CBFA1	*	*	*	*	*	*

**Table 3.1:** The role of recombinant Smad1 N domain, P domain, and C domain in the differentiation of C3H10T1/2/C3H10T1/2<sub>BMP2</sub> cells into osteogenic lineage. ↑ = stimulation or induction; ↓ = repression or reduction; \* = no effect.

The Smad C domain has been shown to have effector function in transcriptional assays (Liu et al. 1996) and *Xenopus* mesoderm forming assays (Baker and Harland 1996). Mutations of carboxy-terminal serines prevents several Smad1 activation events: association with Smad4, accumulation in the nucleus and gain of transcriptional activity (Macias-Silva et al. 1996). All these indicate that the C domain of Smad proteins has played the key role in Smads-mediated signal transduction. In our experiments, the Smad1 C domain induced (in C3H10T1/2 cells) or stimulated (in C3H10T1/2<sub>BMP2</sub> cells) alkaline phosphatase activity and the expression of osteogenic marker genes: osteocalcin, PTH/PTHrP receptor, and collagen I (Fig. 2.9, 2.10, 2.13, 2.14 and 2.15), substantiating the above conclusions. However the



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point mutant Smad1<sup>G419S</sup> has been shown to interfere with BMP-mediated phosphorylation of the C-terminal domain and to exert dominant negative functions (Pamela et al. 1995). Here expression of this Smad1 mutation repressed the BMP2-dependent alkaline phosphatase activity, but not in a dominant-negative way (Fig. 2.8), demonstrating that probably also other molecules than Smad1 are involved in osteo/chondrogenic development.

The sequence-specific DNA-binding activity of the *Drosophila* Mad N domain directed transcriptional activation in response to the *Drosophila* BMP-2 homologue *Decapentaplegic* (DPP) (Kim et al. 1997). This may provide an explanation for the stimulatory effect of Smad1 N domain on the PTH/PTHrP receptor and the osteocalcin gene expression in C3H10T1/2 cells (Fig. 2.14 and 2.13). On the other hand, we also observed that Smad1 N domain is a negative regulator of the alkaline phosphatase activity in C3H10T1/2<sub>BMP2</sub> cells and also repressed collagen I expression in both cell lines (Fig. 2.10 and 2.15). It would be reasonable to hypothesize that in these cases the Smad1 N domain specific binding sites are not sufficient for transcriptional activation.

For the P-domain it has been shown that it contributes to the maximal activity of the Smad1 C domain (Meersseman et al. 1997). Also, the DNA-binding activity of the N domain can be enhanced by the linker region (Kim et al. 1997). We have observed that the stimulation of the osteocalcin, PTH/PTHrP receptor, and collagen I gene expression by Smad1 (C+P) domain and (N+P) domain in C3H10T1/2 cells was higher than by Smad1 C domain and N domain alone (Fig. 2.13, 2.14 and 2.15). This implies that the P domain exerts a biological activity.

The P domain contains several MAPK sites, which can be phosphorylated by the MAP kinase after cell stimulation by EGF, and therefore interferes with nuclear localization of Smad1 and transcriptional activation (Kretzschmar et al. 1997b). But this fact can not explain why the overexpression of the Smad1 P domain interferes with BMP2-induced alkaline phosphatase activity and blocks the expression of osteocalcin and PTH/PTHrP receptor gene in C3H10T1/2<sub>BMP2</sub> cells (Fig. 2.10; 2.13 and 2.14) by a dominant negative effect. In addition, Smad1 P domain also reduced the collagen I expression in parental

### **3 Discussion**

C3H10T1/2 cells and C3H10T1/2<sub>BMP2</sub> cells (Fig. 2.15B). In contrast, Smad1 P domain stimulates the osteopontin expression in C3H10T1/2 cells (Fig. 2.17) and mimics the BMP2/TGF- $\beta$  down-regulation of PEX in C3H10T1/2 cells (Fig. 2.32).

The dose-dependent inhibition of the P domain on alkaline phosphatase activity and the expression of two osteogenetic marker genes: osteocalcin and PTH/PTHrP receptor in C3H10T1/2-Smad1 C cells (Fig. 2.23, 2.24, 2.25), combined with the data that BMP2-dependent osteogenesis was also inhibited by Smad1 P domain (Chapter 2.3) lead to the conclusion that Smad1 P domain inhibited the BMP2- and Smad1 C domain-induced osteogenesis. The similar inhibitory effect was not observed in C3H10T1/2<sub>BMP2</sub> cells expressing Smad3 P domain: overexpression of Smad3 P domain in C3H10T1/2<sub>BMP2</sub> cells did not affect the expression of PTH/PTHrP receptor, osteocalcin and alkaline phosphatase gene (Fig. 2.30; 2.31; 2.29). So the P domain-dependent inhibition on BMP2- or Smad1 C domain-induced osteogenesis seems relatively specific for Smad1 P domain.

Fig. 3.2 shows the similarity of the protein sequences between Smad1 and Smad3. The N domain and C domain are conserved, but the sequences between the two P domains show little similarity. In addition, a stretch 31-amino acid is missing in the P domain of Smad3 when compared with the P domain of Smad1.

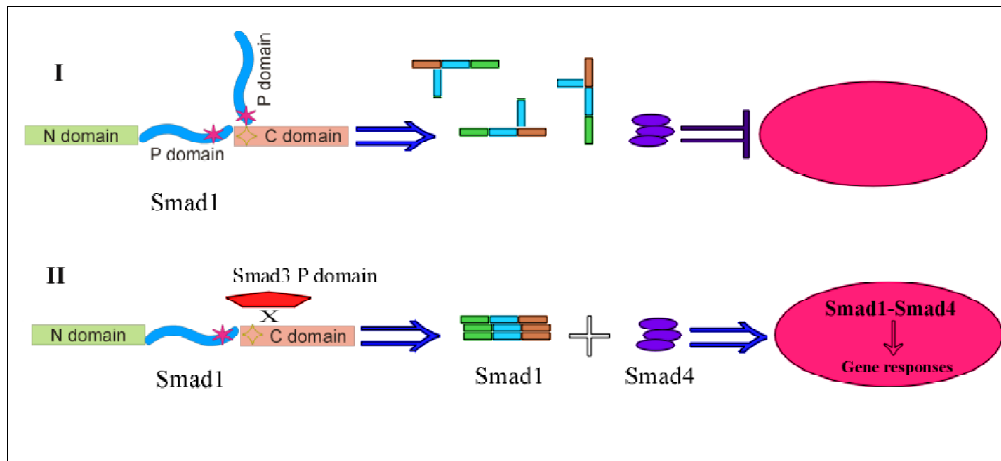
<b>Mu-Smad1</b>	80	MINVTSLSFTSPAVKRLLGWK--QGDEEEKWAEKAVDIALVKKLKKKKGAMEELEKALSCPGRESNCVITIPRSLDGRLOV
<b>Mu-Smad3</b>		***<*****
<b>Mu-Smad1</b>	160	MSSILPTTPIVKRLLGWKKGEQNGQLEKWKCEKAVKSLVKKLK-TGQIDLELEKAITTQNVNTKCTITIPRSLDGRLOV
<b>Mu-Smad3</b>		*****
<b>Mu-Smad1</b>	240	SHRKGLPVITYCRVWRWPDQLQSHHHEKPIECCEYDFGSKQKEVCINPYHYKRVESPVLPVLPVRHSEYNPQHSLLAQFR
<b>Mu-Smad3</b>		*****
<b>Mu-Smad1</b>		SHRKGLPVITYCRVWRWPDQLHSHHHEKRAVELCEFAFNMKKDEVGVNPHYQORVETPVLPVLPVLRHTEIIVET?VLPVPLV
<b>Mu-Smad3</b>		*****
<b>Mu-Smad1</b>		NLEPSEPHNPNATFPDSFQQPNSHPEPHSPNSSYPNSPGSGSTYPHSPASSD?GSPFQIPADTPPPAYMPPEDPRHTEI
<b>Mu-Smad3</b>		*****
<b>Mu-Smad1</b>		PAEFPPPLDDYSHSIPENTNFPAGIEPQSNIPETP-----PPGYLSEGETSDHQ
<b>Mu-Smad3</b>		*****
<b>Mu-Smad1</b>	320	QMTQDNSQEMDTNLMVPNISQDINRADVPRAVAYEEEKHWCSIVYYELNNRVGEAFHASSTSVLVVDGETDPSSNNRNFCLG
<b>Mu-Smad3</b>		*****
<b>Mu-Smad1</b>	400	MNHSMDAGSP--NLSNPMSPAHNNLDLQPVITYCEPAFWCSISISYYELNORVGETFHASOPSNTVDGETDPSN-SERLCLG
<b>Mu-Smad3</b>		*****
<b>Mu-Smad1</b>		LLSNVNRNSTIENTRRHIGKGVHLXYVGSEVYAECLSDSSLFVQSENCNTHHGHPTVCKIPSGCSLKIFENNOEFAQLI
<b>Mu-Smad3</b>		*****
<b>Mu-Smad1</b>		LLSNVNRNMAAVELTRRHIGRGVRLXYTGGEVFAECLSDSAIFVQSENCNQRVGNHPATVCKIPFGCNLIKIFNNOEFAALI
<b>Mu-Smad3</b>		*****
<b>Mu-Smad1</b>		AQSVNHGFEIVYELTKMCTIRMSFYKQWGAHYHRODVTSTPCWIEIHLHGLOWLDKVLITQMGSEHNPISSVS
<b>Mu-Smad3</b>		*****
<b>Mu-Smad1</b>		AQSVNQGFEAVYQLTRMCTIRMSFVKQWGAEXRROTATSTPCWIEIHLHGLOWLDKVLITQMGSEPSIROSSVS
<b>Mu-Smad3</b>		*****
		<b>Smad-N; MH1</b> <b>Smad-P (proline-rich-linker)</b> <b>Smad-C; MH2</b> <b>* by MAPK</b> <b>* by BMP-receptors</b>

**Fig. 3.2:** The Protein sequence alignment between Smad1 and Smad3.

### **3 Discussion**

How Smad1 P domain exerts its function is unclear, but the studies from Lechleider and colleagues with Smad4 indicates that there is a 47-amino acid SAD (Smad Activation Domain) within the COOH-terminal portion of the proline-rich linker region of Smad4 which is necessary for this molecule to transduce signaling responses downstream from TGF- $\beta$  receptors. In the absence of ligand, the N domain of Smad4 may obscure the SAD which locates at the extreme COOH-terminal of the linker region, either by direct blockade of this region or by conformational interference. Following ligand-activation, there may be unfolding of the molecular structure resulting in exposure of the SAD, allowing it to interact with other signaling partners (de Caestecker et al. 1997). Although the linker region of Smad4 shows minimal sequence similarity with those of the other Smad family members, it is still possible that the P domain of Smad1 could also possess a SAD-like domain, which is blockaded in the absence of ligand and is activated after ligand mediated signaling. This potential SAD-activity would be present in overexpressed recombinant P domains.

The function of SAD-like domains is interacting with signaling molecules. In the cells overexpressing Smad1 P domains, the Smad1 P domains could bind to Smad1 C domains through SAD-like domains. This binding interferes with the multimerization of Smad1 C domains, therefore Smad1 C domains could neither form heteropolymers with Smad4 nor enter into the nucleus to exert its nuclear functions (Fig. 3.3 -I). This appears to be a relatively Smad1 specific event since the overexpression of Smad3 P domain does not interfere with the nuclear functions of Smad1 C domains (Fig. 3.3-II).



**Fig. 3.3:** Signal transduction in cells overexpressing (I) Smad1 P domain; (II) Smad3 P domain.

The osteocalcin gene has been reported to have a CBFA1 binding site in 5' flanking region and is induced by the transient expression of *CBFA1* in MC3T3-E1 cells and C3H10T1/2 Cl8 (Ducy et al. 1997). In addition, CBFA1 most likely regulates collagen I gene expression (Komori and Kishimoto 1998). In the present study, the results showed that the expression of *CBFA1* in C3H10T1/2 and C3H10T1/2<sup>BMP2</sup> cells recombinantly expressing Smad1 domains was kept in the same level in comparison with control cells (Fig. 2.16), whereas the expression of osteocalcin and collagen I was obviously regulated in above cell lines (Fig. 2.13 and 2.15). These data correlate well with these studies that BMP2 does not enhance the *CBFA1* expression although BMP7 (Ducy et al 1997) or BMP4/BMP7 heterodimer (Tsuji et al 1998) specifically enhances expression of *CBFA1* in C3H10T1/2 cells, MC3T3-E1 cells, and C2C12 cells. This implies that the essential factor for osteoblast differentiation-CBFA1 is not up-regulated by BMP2 or the Smad1 signal transduction.

#### **3.3 The transcriptional assays substantiated the genetic and histological analyses of Smad1 signaling mediator**

TGF- $\beta$  family members mediate their cellular actions at least in part by controlling the transcription of target genes. Investigation of transcriptional actions by Smads has revealed a complex pattern involving multiple proteins and diverse response promoter elements. For instance, the C domain fused to a heterologous DNA binding domain, was found to induce a transcriptional response (Liu et al. 1996; Meersseman et al. 1997). In addition, Smad2 and Smad4 participate in the activin-mediated transcriptional induction of the *Xenopus Mix-2* promoter where they act as co-activators via an interaction with the FAST-1 DNA-binding factor (Chen, X. et al. 1996; 1997). Furthermore, the *Drosophila* Mad, deleted in the conserved MH<sub>2</sub> domain, binds directly to GC-rich regions of various enhancers (Kim et al. 1997).

Recently, the studies of this complex mechanism of transcriptional regulation of TGF- $\beta$  responsive genes lead to precise location of the Smad binding sites in promoter sequences, an 8 bp palindromic DNA sequence 5'-GTCTAGAC-3' was identified as the Smad binding element (SBE) through PCR-based random oligonucleotide selection process. The 4 bp site 5'-GTCT-3' in SBE represents the minimal binding sequence, was termed as Smad box (Zawel et al. 1998). Almost at the same time, it is reported that the identification of the SBEs composed of the sequences 5'-CAGACA-3' in the promoter of the JunB gene (Jonk et al. 1998). This Smad3/Smad4 binding sequences were also identified within the promoter of the human plasminogen activator inhibitor-1 (PAI-1) gene and was termed CAGA boxes (Dennler et al. 1998). The SBE is only efficiently bound by bacterially produced Smad3 and Smad4, but not Smad2 and BMP signaling Smad1 and Smad5 proteins (Dennler et al. 1998; Jonk et al. 1998). It is possible that Smad1 and Smad5 require additional proteins for high affinity binding to these sequences. Alternatively, in contrast to Smad3, these Smads may associate with their target sequence only through Smad4 (Jonk et al. 1998). The full-length Smad4 protein possessed a direct DNA-binding activity on the CAGA box (Dennler et al. 1998) suggesting a mechanism other than only the N domain of Smad1 is able to bind DNA.

### **3 Discussion**

The two modes of Smad function, as direct DNA binding proteins and as transcriptional coactivator proteins, broaden the possible range and diversity of Smad promoter targets. In certain contexts, the interaction of specific Smad heteromers with specific transcription factors may lead to promoter activation. In this context, the DNA binding function of the Smads becomes dispensable. While other promoter contexts may require the DNA binding function of the Smads to target and activate the promoter, as is the case with Mad and the vestigial promoter (Yingling et al. 1998).

We investigated the individual effect of Smad1 and its domains on the activity of BMP-responsive gene promoter-luciferase constructs, such as Msx1, PTH/PTHrP receptor, and JunB. Our studies show that BMP2 background stimulates the activity of three Msx1 promoter luciferase constructs in C3H10T1/2 cells. Among the Smad1 wt and its domains, the Smad1 C domain maximally stimulated the Msx1 -164/+102 promoter luciferase activity (Fig. 2.51, 2.52, 2.53), which implies that the Smad1 regulates the Msx1 transcription not necessarily by the DNA binding activity of Smad1 N domain, but by the transcriptional activity of Smad1 C containing domains. Because the DNA binding activity of Smad C domain has not been reported, it is therefore reasonable to speculate that the Smad1 C domain or Smad1 wild type might form a complex with additional proteins, possibly with Smad4, which alone is able to bind DNA in vitro. This DNA binding ability is likely to be regulated or modified by association with its Smad partners in response to exogenous signals under physiological conditions (Yingling et al. 1997), which then bind to the specific DNA sequence in the Msx1 -164/+102 promoter region and activate its transcription.

The Smad1 wild type in our studies was also able to stimulate the Msx1 -164/+102 and PTH/PTHrP receptor promoter luciferase activity (Fig. 2.51; 2.53; 2.48). This result is in contrast with the very weak induction on the LacZ reporter expression by the Smad1 wild type in HeLa cells (Meersseman et al. 1997), but consistent with the transcriptional activity of Smad1 full length induced by BMP treatment (Liu et al. 1996) and activation of JunB transcription in vivo by Smad1 and Smad4 (Jonk et al 1998) .

### **3 Discussion**

Interestingly, also the Smad1 P domain stimulated the promoter luciferase activity in comparison with the control vector. This phenomenon was not only observed in the Msx1 -164/+102 promoter assay (Fig. 2. 51; 2.53) but also in the JunB (Fig. 2.44) and PTH/PTHrP receptor (pTK 0.6) (Fig. 2. 48) promoter assay. This observation is consistent with a weak but detectable transactivatory activity of the P domain of Smad1 (Meersseman et al. 1997). In combination with C domain, however, the P domain exerts a clear inhibitory effect.

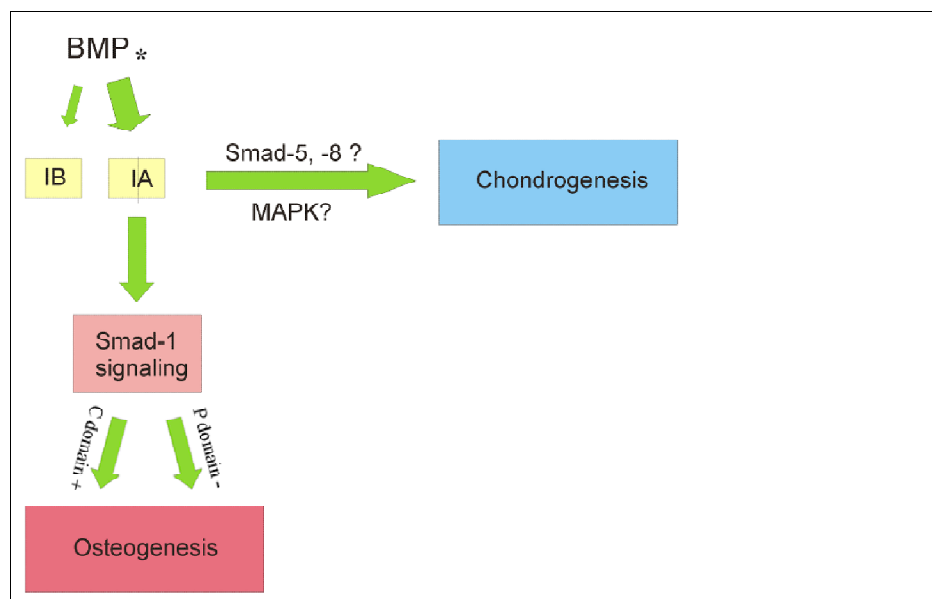
The fact that Smad1 P domain interferes with the stimulation of Smad1 C domain on the promoter luciferase activity of Msx1 -164/+102, JunB, and PTH/PTHrP receptor (pTK0.6) (Fig. 2.53, 2.44, 2.48) raises the possibility that Smad1 P domain could form a complex with Smad1 C domain, the complex might be unable to enter into the nucleus and therefore the P domain would weaken the transcriptional stimulation of the Smad1 C domain on the BMP-responsive genes.

The PTH/PTHrP receptor promoter pTK0.6 luciferase activity was stimulated by the Smad1 wild type and C domain in our assays ( Fig. 2.48), suggesting that a Smad1 responsive element exists in this promoter region. The activity of this 0.6 kb promoter fragment, however, could be silenced by other regions of the promoter, since the PTH/PTHrP receptor promoter luciferase constructs pLuc S3.9 and pLuc S2.9 were not regulated by the Smad1 domains.



### **3.4 The model for signaling cascades of BMP-mediated osteo-/chondrogenic development in C3H10T1/2 cells**

Based on our results, a model for signaling cascades in BMP-mediated osteo/chondrogenic development in C3H10T1/2 cells is presented (Fig. 3.4). The BMPs exert their functions through the association with type I and type II receptors. BMP2/BMP4 bind two human type I receptors, Alk3 and Alk6 (Lin et al. 1992), which have also been termed BMP receptor type IA and type IB (BMPR-IA and BMPR-IB) (ten Dijke et al. 1994). BMPR-IA is predominantly responsible for the BMP-dependent osteo/chondrogenesis. BMPR-IA seems to mediate induction of osteogenesis through the signaling mediator Smad1, while the chondrogenic induction could be via the Smad5, Smad8 or MAPK signaling pathway (Ph D thesis, Christian Kaps).



**Fig. 3.4:** Signaling cascades for BMP-mediated osteo/chondrogenic development in C3H10T1/2 cells. \* based on investigations of Christian Kaps in the laboratory.

## 4 Summary

The bone morphogenetic proteins (BMPs) belong to the transforming growth factor  $\beta$  (TGF- $\beta$ ) superfamily. They regulate biological processes not only in bone morphogenesis, but also the development of all organs and tissues, as well as in the establishment of the basic embryonic body plan. The Smad-family members have been characterized recently as important signaling mediators for TGF- $\beta$ -related factors. Smad1, Smad5 and Smad8 have been identified as BMP signal transducers. The aim of this research was elucidate the functions of the three domains: the N-terminal domain, the C-terminal domain and the proline-rich linker domain of Smad1, especially the function of the less conserved proline-rich linker region, in the mesenchymal differentiation processes, particularly in regard to osteo-/chondrogenic development. For this purpose eukaryotic expression vectors harbouring the *Xenopus* Smad1 wild type, the C domain, the (C+P) domain, the N domain, the (N+P) domain, or the P domain was permanently transfected into the murine mesenchymal progenitor cell C3H10T1/2 and BMP2 expressing C3H10T1/2 cells.

The differentiation potential of C3H10T1/2 and C3H10T1/2<sub>BMP2</sub> cells expressing recombinant Smad1 or its domains was investigated by histological staining and also by RT-PCR analyses of the expression of several marker genes, which are specific or typical for osteogenic-, chondrogenic-, and adipogenic development. The following results were obtained:

- Smad1 and its biological active C domain initiated osteogenic development in the C3H10T1/2 cells and stimulated in C3H10T1/2<sub>BMP2</sub> cells differentiation into osteogenic lineages.
- The N domain was sufficient to initiate transcription of osteogenic marker genes such as the osteocalcin or PTH/PTHrP receptor gene in parental C3H10T1/2 cells. In general, however, this domain blocked osteogenic development when recombinantly expressed in C3H10T1/2<sub>BMP2</sub> cells.

- The P domain dominant-negatively inhibited the BMP2-dependent osteogenesis in C3H10T1/2 cells. C3H10T1/2-Smad1 C cells expressing different amount of expression vector harbouring Smad1 P domain showed that the P domain of Smad1 inhibited osteogenesis in a dose dependent manner.
- C3H10T1/2<sub>BMP2</sub> cells expressing recombinant Smad3 P domain revealed that the inhibitory effect of P domain on osteogenesis is not a common property of all Smads, but seems specific for Smad1.
- Smad1 and its domains do obviously not participate in the BMP2-dependent chondrogenic development, since they could neither differentiate C3H10T1/2 cells into chondrocytes nor was the chondrogenic potential of C3H10T1/2<sub>BMP2</sub> cells influenced by Smad1 or its domains.
- Adipogenic development was affected by Smad1 or its domains in C3H10T1/2 cells, but other factors, appears to complement Smad1 in this process.
- The transcriptional assays of promoter luciferase constructs for some BMP-responsive genes, such as PTH/PTHrP receptor, JunB, and Msx1, substantiated the histological and genetic analyses of Smad1 function by showing stimulation of the promoter luciferase activities by the Smad1 C domain.
- The interference of the Smad1 P domain with Smad1 C domain-dependent stimulation of luciferase activity suggests that the Smad1 P domain might form a complex with the Smad1 C domain and thereby inhibits the translocation of Smad1 C domain into the nucleus.

The analysis of effects of the Smad1 domains has important value in understanding the process of BMP2-dependent osteo-/chondrogenic development in mesenchymal progenitors (C3H10T1/2).

## **5 Methods**

### **5.1 General methods**

#### **5.1.1 Frequently used buffers and solutions**

TE(10/0,1)	10 mM Tris-HCl (pH7.5) 0.1 mM EDTA
25 x TA	1 M Tris-HCl (pH8.0) 50 mM EDTA
PBS: (Phosphate Buffered Saline)	140 mM NaCl (pH 6.8- 7.0) 27 mM KCl 7.2 mM Na <sub>2</sub> HPO <sub>4</sub> x 2 H <sub>2</sub> O 14.7 mM KH <sub>2</sub> PO <sub>4</sub>

#### **5.1.2 Sterilization**

Glassware was sterilized for 2 hours at 180°C. Plasticware and most of the solutions were autoclaved. The solutions which can not be autoclaved were sterilized by filtration.

### **5.2 General methods of DNA cloning (Maniatis, 1989)**

#### **5.2.1 DNA digestion with restriction endonucleases**

Digestion of DNA with restriction endonucleases was carried out by incubating the enzyme(s) with DNA using appropriate reaction conditions. The amounts of enzyme and DNA, buffer and ionic concentrations, and the temperature and duration of the reaction varied depending upon the specific application. Most of the restriction enzymes are active at 37°C. Under optimal conditions one unit (1 U) enzyme cleaves 1 µg DNA within one hour. The reaction was stopped by incubating for 15 min at 65°C. Heat resistant enzymes were inactivated by extraction with phenol, DNA was precipitated from the aqueous phase by ethanol.

Buffer for *Bam*HI: NEBuffer *Bam*HI

150 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 1 mM DTT  
(pH 7.9). Supplement with 100 µg/ml BSA.

Buffer for *Bgl* II: NEBuffer 3

100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 1 mM DTT  
(pH 7.9).

Buffer for *Eco*RI: NEBuffer *Eco*RI

50 mM NaCl, 100 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 0.025% Triton  
X-100 (pH7.5).

Buffer for *Hind* III: NEBuffer 2

50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 1 mM DTT  
(pH7.9)

### 5.2.2 Agarose gel electrophoresis of DNA

Agarose gels of appropriate concentration (from 0.5% to 2.0%) were used to separate DNA fragments of different sizes. Agarose was melted in 100 ml 1x TA-running buffer (400 mM Tris-acetate; 20mM EDTA, pH 8) in a microwave oven and was swirled to ensure even mixing. The final concentration of ethidium bromide incorporated in gel and electrophoresis buffer was 0.5 µg/ml. The melted agarose should be cooled to 55°C before pouring into the gel chamber with gel comb. After the gel was hardened, the gel chamber was placed in the electrophoresis tank which was filled with 1x TA running buffer until the buffer covered the gel to a depth of 1 mm. The samples together with an appropriate standard for size determination were mixed 10x loading buffer (20% Ficoll; 0.1% Orange G; in TE) and added to the sample wells. The ideal running condition is 5 Volt per cm.

Agarose gel	Effective range of resolution for linear DNA fragments
0.5 %	0.8 - 15 kb
1.0 %	0.4 - 8 kb
1.2%	0.2 - 6 kb
1.5%	0.2 - 4 kb
2.0%	0.1 - 3 kb

### 5.2.3 DNA and RNA standards

DNA standards:

Lambda DNA /*Eco*RI+ *Hind*III Marker:

21226, 5148, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564 bp

pBR322 DNA/*Mva*I( *Bst*NI) Marker:

1857, 1058, 929, 383, 121 bp

100bp DNA Ladder:

1000, 900, 800, 700, 600, 500, 400, 300, 200, 100, 80 bp

pM2 DNA/*Hae*III Marker:

1180, 1760, 1410, 860, 845, 672, 615, 525, 333, 295, 272, 167, 152, 120,  
95, 49, 44 bp

RNA standard:

CA3103(BRL):

9.5, 7.5, 4.4, 2.4, 1.4, 0.24 KB

### 5.2.4 Isolation of DNA fragments from low melting point agarose gel

(The chemicals are supplied by "JET-SORB" kit; *GENOMED*)

This method can be used to purify and isolate DNA fragments after separation by an LMP (Low Melting Point)-agarose gel. The target band was cut with a scalpel from the 1% LMP gel and weighed. 300 µl Buffer A1 and 10 µl JETSORB Solution were added to per 100 mg gel. The mixture was incubated at 50°C for 15 min to allow the gel to melt and DNA to bind to the resin in the JETSORB Solution. After 30 sec at 15 000 x g centrifugation, the supernatant was discarded and the resin was resuspended in Buffer A1. In the same way the resin was washed two times with Buffer A2. The resin was air-dried, the DNA was eluted with an appropriate volume of TE at 50°C for 5 min, centrifuged as before, and the supernatant containing DNA was saved.

Buffer A1: conc. NaClO<sub>4</sub>; TBE; NaAc

Buffer A2: EtOH; NaCl; EDTA; Tris/HCl

### **5.2.5 Isolation of DNA fragments from standard agarose gels**

(The chemicals are supplied by High Pure PCR Product Purification Kit; Boehringer Mannheim)

The DNA fragments can also be purified and isolated from standard agarose gels. The target band was cut from the gel and put into an eppendorf tube, and the weight was determined. 300 µl Binding Buffer (3 M guanidinium-thiocyanate, 10 mM Tris-HCl, pH 6.6, 5% ethanol) was added per 100 mg gel, incubated at 56°C for 15 min. To ensure that the gel is totally melted in the Binding Buffer the mixture was vortexed every 2-3 min. 150 µl isopropanol was added per 100 mg gel, followed by 10 sec vortexing. A filter tube was put onto an accepting tube and the DNA solution was pipetted into the filter tube. After 30 sec centrifuging at 13 000 g, the filter tube was washed with 500 µl Washing Buffer (20 mM NaCl, 10 mM Tris-HCl, pH 7.5) and 200 µl Washing Buffer, respectively, as before. For elution of bound DNA, the filter tube was put onto a clean eppendorf tube, 50-100 µl TE was added. After centrifuging, the DNA solution was available for further analysis.

### **5.2.6 Purifying and concentrating of DNA from aqueous solutions**

Phenol extraction or ethanol precipitation were often used to remove proteins and solute molecules from aqueous solutions, or to concentrate DNA solutions. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the DNA solution. The mixture was vortexed vigorously for 10 sec and microcentrifuged 45 sec at RT. The top (aqueous) phase containing DNA was carefully transferred to a new tube. 1/10 volume of 3 M sodium acetate (pH 5.2) was added to the DNA solution, mixed briefly. 2 to 2.5 volumes of ice-cold 100% ethanol was added to the mixture from which DNA was precipitated at -70°C for 15 min to 1 hr.

The sample was centrifuged for 15 min at high speed. 1 ml 70% ethanol was added to the pellet. The tube was inverted several times, followed by centrifugation. The pellet was air-dried and was dissolved in an appropriate volume of TE buffer (pH 8.0) and stored at -20°C.

### **5.2.7 Removal of low-molecular-weight oligonucleotides and triphosphates**

Using ammonium acetate in place of sodium acetate allows the preferential precipitation of longer DNA molecules. Therefore, the small single- or double-stranded oligonucleotides can be removed from DNA solutions.

An equal volume of 4 M ammonium acetate (pH 4.8) was added to the DNA solution, and mixed well. Then 2 volumes of ice-cold 100% ethanol were added, vortexed 10 sec and processed as described in 5.2.6.

### **5.2.8 Quantification of nucleic acids**

#### **5.2.8.1 Measurement of absorbance**

The concentration-dependent absorbance of nucleotides at 260 nm can be used to determine the concentration of DNA or RNA solutions. The nucleic acid solution was diluted 1:50 or 1:100 with water. 100  $\mu$ l (water as a reference) was added into a quartz cuvette, absorbance was measured at 260 and 280 nm. 1 OD<sub>260</sub> equals 50  $\mu$ g/ml double-stranded DNA or 40  $\mu$ g/ml single-stranded DNA or RNA. A carefully prepared nucleic acid solution has a ratio of OD<sub>260</sub>/OD<sub>280</sub> between 1.5 and 2.0.

#### **5.2.8.2 Qualification in agarose gels**

Alternatively to the method described above, the concentration of nucleic acids can be determined by staining the agarose gel with ethidium bromide. A defined amount of marker was used as a standard to run a gel in parallel with the DNA in question. The gel was documented with a digital camera and analysed with appropriate software (WINCAM from CYBERTECH).

### **5.2.9 Dephosphorylation of DNA**

Alkaline phosphatase (Shrimp Alkaline Phosphatase, USB) was used to dephosphorylate the 5'-termini of DNA-ends in order to prevent vector self-ligation



5 µg DNA were mixed with 5 U alkaline phosphatase in 1x alkaline phosphatase buffer and incubated at 37°C for 1 hr. The reaction was stopped by heating to 65°C for 10 min or extracting with phenol.

10 x alkaline phosphatase buffer:

200 mM Tris-HCl, pH 8.0

100 mM MgCl<sub>2</sub>

### **5.2.10 Ligation of DNA fragments**

The Ready-To-Go T4 DNA Ligase Kit (Pharmacia) was used to ligate DNA fragments. T4 DNA ligase catalyzes the repair of single-stranded nicks in duplex DNA and joins duplex DNA restriction fragments having either blunt or cohesive ends. It is the only ligase that efficiently joins blunt-end termini under normal reaction conditions.

The vector was digested with restriction enzymes and dephosphorylated as described before, and the DNA insert was also digested with appropriate restriction enzymes to get compatible ends. For sticky-end ligation, the ratio of vector to insert DNA was 1:1 to 1:2 (total DNA is 200 ng to 500 ng). The dry ligation mixture was dissolved with 20 µl DNA solution of vector and insert. The ligation sample was incubated at 16°C overnight. For blunt-end ligations, the ratio of vector to insert DNA was 1:1 to 1:4 (total DNA is 200 ng to 1000 ng). Blunt-end ligations were performed at RT.

After ligation, the mixture was precipitated with 0.5 vol. NaCl and 0.6 vol. Isopropanol for 1 hr at -70°C, afterwards centrifuged at 4°C and 20 000x g. The pellet was washed 2 times with 70% ethanol and dissolved in TE.

The dry ligation mixture:

6 U Weiss T4 DNA ligase

66 mM Tris-HCl, pH 7.6

6.6 mM MgCl<sub>2</sub>

0.1mM ATP, 0.1 mM Spermidine, 10 mM DTT.

## **5.3 Work with *E.coli***

### **5.3.1 *E.coli* line**

*E.coli* SURE (Stop Unwanted Rearrangement Events), (Greener, 1990):

recB, recJ, sbcC201, uvrc, umuC: Tn5 (kan<sup>R</sup>), mcrA, mcrB, mrr lac, Δ(hsdRMS), endA1, gyrA96, thi, relA1, supE44{F', proAB, lacIqZ Δ M15 FN10 (tet<sup>R</sup>)}

### 5.3.2 Culture media:

#### LB-Medium:

Bacto-Trypton	10 g/l
Bacto-yeast extract	5 g/l
NaCl	5 g/l

#### TB-Medium:

Bacto-Trypton	12 g/l
Bacto-yeast extract	24 g/l
87 % Glycerol	4 ml
KH <sub>2</sub> PO <sub>4</sub>	17 mM
K <sub>2</sub> HPO <sub>4</sub>	72 mM

#### SOB-Medium:

Bacto-Tryptone	2 %
Bacto-yeast extract	0,5 %
NaCl	10 mM
KCl	2.5 mM
MgCl <sub>2</sub>	10 mM
MgSO <sub>4</sub>	10 mM

#### SOC-Medium:

SOB-Medium with 20 mM Glucose

#### Agar Plates:

7.5g/l Bacto-Agar in LB-medium

#### Ampicilline:

working concentration: 50 mg/l; stock solution: 25 mg/ml in 70 % Ethanol

#### Tetracycline:

working concentration: 5 mg/l; stock solution: 5 mg/ml in 100 % Ethanol

#### Kanamycin:

working concentration: 50 mg/l; stock solution: 50 mg/ml in water

### **5.3.3 Conservation of bacterial strains (Maniatis et al, 1989)**

A bacterial strain can be stored on a culture plate for 2-4 weeks at 4°C. For permanent storage, 1 ml overnight culture of the desired strain was mixed with 1 ml autoclaved glycerol (87 %) and was kept at -70°C.

### **5.3.4 Preparation of competent cells (Dower et al, 1988)**

A single clone of *E.coli* SURE was incubated in 2 ml LB medium with tetracycline overnight at 37°C with moderate shaking. This preculture was transferred into 1000 ml LB medium in a sterile 2-liter flask (which allows for sufficient aeration) and cultured at 37°C with moderate shaking for 2 to 5 hr until an OD<sub>600</sub> of 0.6 to 0.7 was reached.

The cells were aliquoted into four 250-ml flasks and incubated on ice for 30 min. The cells were centrifuged 15 min at 1500 x g, 4°C. Each pellet was resuspended thoroughly but gently in 250 ml ice-cold sterile deionized water. The cells were centrifuged again, and washed with 100 ml ice-cold sterilized water. After centrifugation the pellets were pooled with 10 ml ice-cold sterile glycerol (10%; w/v) and centrifuged again. The pellet was resuspended with 2 ml glycerol. All steps should be performed on ice. The cells were dispensed into pre-chilled 1.5 ml eppendorf tubes, and were frozen immediately in liquid nitrogen. The competent cells were stored at -70°C.

### **5.3.5 Transformation by high voltage electroporation**

Electroporation with high voltage is currently the most efficient method for transforming *E. coli* with plasmid DNA. Competent cells were thawed on ice. 1 µl of the DNA solution to be transformed was added to 50 µl competent cells, mixed well, and incubated for 1 min on ice. The transformation mixture was transferred into an electroporation cuvette (gap width: 0.2mm) and pulsed at 2.5 kV/25 µF/200 Ohm. 1 ml pre-warmed SOC medium was added immediately afterwards. The cells were transferred into a sterile culture tube, and incubated 1 hr with moderate shaking at 37°C. The transformation culture was spread on LB plates containing appropriate antibiotics and incubated at 37°C overnight.

### **5.3.6 Identification of recombinant bacterial clones by PCR**

PCR (Polymerase Chain Reaction) can be used to check for positive clones if there are specific primers available. Single clones were picked with a tooth-pick and transferred into 20 µl TE as well as onto a master-plate. The master plate was incubated at 37°C. PCR was performed with 50% of the DNA solution as template. PCR products were checked by gel electrophoresis.

### **5.3.7 Isolation of plasmid DNA from E. coli (Birnboim and Doly 1979)**

#### **5.3.7.1 Minipreps of plasmid DNA**

This is the most commonly used protocol for isolation of small quantities of plasmid DNA from bacterial cells. Bacterial cells were lysed by treatment with a solution containing SDS and NaOH (SDS denatures bacterial proteins, and NaOH denatures chromosomal and plasmid DNA). The mixture was neutralized with potassium acetate, causing the covalently closed plasmid DNA to reanneal rapidly whereas most of the chromosomal DNA and bacterial proteins remained precipitated and could be removed by centrifugation. The reannealed plasmid DNA from the supernatant was then concentrated by ethanol precipitation.

Single clones were picked for simultaneous inoculation of a master-plate and a 2 ml-culture, both of which were incubated overnight at 37°C. The next day the overnight cultures were transferred into a 2 ml eppendorf tube and centrifuged by 8000 rpm for 1 min. The cell pellet was resuspended in 200 µl GTE solution (Glucose 50 mM, Tris-HCl 25 mM, pH 8.0, EDTA 10 mM, 4 mg/ml Lysozym, optional) and incubated for 5 min at RT. 400 µl of freshly prepared denaturing-solution (NaOH 0.2 N, SDS (w/v) 1 %) was added and incubated for 5 min on ice. 300 µl 7.5 M ammonium acetate was added and incubated again on ice for 10 min. Cell debris and chromosomal DNA were pelleted at 13 000 x g for 10 min. The supernatant was transferred to a new tube, mixed with 0.6 vol. isopropanol, and placed for 15 min at -70°C to precipitate nucleic acids. A 10 min centrifuging at 13 000 x g pelleted plasmid DNA. The pellet was washed with 1 ml of 70 % ethanol, air-dried, and dissolved in 100 µl TE.

To confirm whether the clone contained the desired insert, 10 µl of the DNA was used for a restriction digest with appropriate enzymes.

### **5.3.7.2 Maxi prep of plasmid DNA**

**(The chemicals are supplied by JETSTAR kit, GENOMED Inc.)**

JETSTAR is a new and unique anion exchange resin developed by GENOMED. The kit was designed to combine binding of DNA to columns with a modified alkaline/SDS lysis procedure for the preparation of plasmid DNA. The JETSTAR-purified plasmid DNA is reported to be of a higher quality than 2 x CsCl-purified plasmid DNA.

Bacterial colonies harboring the desired recombinant plasmid were cultivated in 2-3 ml LB with appropriate antibiotics at 37°C with vigorous shaking for 7-9 hr. 1-2 ml of the preculture was transferred into 250-500 ml LB medium with antibiotics (in a 2-Liter flask) and incubated at 37°C with vigorous shaking overnight.

The cells were collected by 10 min centrifuging at 4°C, 6000 x g. The pellet was resuspended in 10 ml Cell Resuspending Solution (50 mM Tris-HCl, pH 8.0, 10 mM EDTA) until homogeneous. 10 ml Cell Lysis Solution (200 mM NaOH, 1 % SDS (w/v)) was added to the suspension and mixed gently. The mixture was incubated for 5 min at RT. Then 10 ml Neutralization Solution (3.2 M potassium acetate/ acetic acid, pH 5.0) was added and mixed immediately but gently. The mixture was centrifuged for 15 min at 20°C, 15 000 x g. The supernatant was applied onto the column, which had been equilibrated by Column Equilibration Solution (600 mM NaCl, 100 mM sodium acetate/ acetic acid, pH 5.0, 0.15 % Triton X-100). The column was washed with 60 ml Column Washing Solution (800 mM NaCl, 100 mM sodium acetate/ acetic acid, pH 5.0). Then pre-warmed (50°C) DNA Elution Buffer (1250 mM NaCl, 100 mM Tris-HCl, pH 8.5) was added to the column, the eluate was collected in a 30 ml Corex tube. 0.7 vol. isopropanol was added to precipitate DNA and kept at -70°C for 15 min. The DNA solution was centrifuged for 30 min at 4°C, 15 000 x g. The pellet was washed 2 times with 5 ml 70 % ethanol each and recentrifuged. The pellet was air-dried and dissolved in 1ml TE.

**5.4 Cloning of cDNAs (Frohman et al. 1990)****5.4.1 Reverse transcription of mRNA**

(SuperScript™ RNase H<sup>-</sup> Reverse Transcriptase kit, GIBCO-BRL, 18053-017)

SuperScript™ RT RNase H Reverse Transcriptase is produced from a cloned M-MLV RT gene from which the RNase H sequence has been deleted, reducing the RNase H activity to nondetectable levels. The enzyme can be used to synthesize first strand cDNA and will generally result in higher yields of cDNA and more full length products.

5 µg total RNA or 0.5 µg mRNA and 1 µl Oligo (dT)<sub>12-18</sub> (500 µg/ml)<sup>a</sup>, which binds to the 3'-poly(A)-end of mRNA, were mixed in a 1.5 ml nuclease-free eppendorf tube, water was added to a final volume of 12.6 µl. The mixture was heated to 70°C for 10 min and quickly chilled on ice. The contents of the tube were collected by brief centrifugation. Then 7.4 µl reaction solution was added to the tube and mixed by gently vortexing. The tube was equilibrated at 37°C for 2 min. 1 µl (200 U) of SuperScript RT was added to the mixture, mixed gently, and incubated at 37°C for 30-60 min. 30 µl TE was added to the mixture and the reaction was terminated by heating to 90 °C for 5 min followed by quickly chilling on ice.

reaction solution:

5 x First Strand Buffer	4 µl
0.1 M DTT	2 µl
dNTP stock (10mM)	1 µl
RNasin <sup>R</sup> RNase Inhibitor	0.4 µl ( 16 U)

5 x first strand buffer:

Tris-HCl, pH 8.3	250 mM
KCl	375 mM
MgCl <sub>2</sub>	15 mM

**5.4.2 PCR (Polymerase-Chain-Reaction)-Amplification**

Reverse transcription of RNA followed by the Polymerase Chain Reaction (RT-PCR) is an extremely sensitive method to amplify specific DNA fragments of interest from a mixture, or to detect and quantify specific mRNAs, respectively.

Traditionally, levels of individual mRNAs have been analyzed by procedures such as Northern blots, RNA dot/slot blots, nuclease protection and *in situ* hybridization. Application of the polymerase chain reaction technique provides another method of mRNA analysis. This PCR-based method has been variously termed RNA-PCR (Kawasaki et al., 1991), RT-PCR (Rappolee et al., 1991), RNaphenotyping (Chelly et al., 1988), and Message Amplification Phenotyping (MAPPING) (Brenner et al., 1989). The RT-PCR method has become increasingly popular for analysis of gene transcripts, primarily because it is highly sensitive and rapid.

Basic PCR components include reaction buffer, dNTPs, primers, cDNA template and a thermostable DNA polymerase. Taq Polymerase works at different  $Mg^{2+}$  concentrations and different temperatures (the optimum temperature is 72°C). Moreover heat resistance even at 94°C allows to change the temperature, in order to place the molecule under the best conditions of amplification possible. The primers used should exactly match the cDNA template and flank the 5' and 3' ends of the template DNA. The samples are analyzed by agarose gel electrophoresis.

Standard conditions for PCR amplification:(total volume: 20µl)

Template DNA	0.1 ng/µl
Nucleotide	250 µM
Taq-Polymerase	0.025 U/µl
MgCl <sub>2</sub>	2.5 mM
10 x PCR Buffer	2µl
Primer (5' + 3')	0.1 pmol/µl

The standard PCR program:

Precycle:

94°C            1 min

Followed by about 30 cycles:

94°C	20 sec	Denaturing of DNA
X °C	20 sec	Primer annealing (the temperature is primer-pair specific)

72°C	30 sec	Elongation of DNA
Final cycle:		
72°C	3 min	Completion of DNA-fragments

10 x PCR Buffer:

KCl	500 mM
Tris-HCl, pH 8.3	100 mM
gelatin	0.01%
dNTP- Mixture:	
dATP	10 mM
dGTP	10 mM
dCTP	10 mM
dTTP	10 mM

### 5.4.3 Cloning of PCR Products

(All chemicals are from Expand™ High Fidelity PCR System, Boehringer Mannheim)

In this protocol, synthetic oligonucleotides incorporating restriction sites are used to amplify a region of DNA to be subcloned into a vector containing compatible restriction sites. The Expand™ High Fidelity PCR System, is composed of a unique enzyme mix containing thermostable Taq DNA and Pwo DNA polymerases (Bernes et al., 1994). This powerful polymerase mix is designed to give PCR products with high yield, high fidelity and high specificity from episomal and genomic DNA. Due to the inherent 3'-5' exonuclease proofreading activity of Pwo DNA polymerase this system results in increased accuracy of DNA synthesis ( $0.85 \times 10^{-5}$  error rate) compared to Taq polymerase ( $2.6 \times 10^{-5}$  error rate).

Two master mixes are set up as follows (all on ice):

Master mix 1 (25 µl):

dNTPs	1 µl	final concentration 200µM
Primer (3'+5')	3 µl	final concentration 300nM
Template DNA	10-100 ng	
sterile redist. H <sub>2</sub> O	up to 25 µl	

Master mix 2 (25µl):

10 x Expand HF buffer with 1.5 mM MgCl <sub>2</sub>	5 µl
Expand™ High Fidelity PCR System enzyme	0.75 µl (2.6 U)



sterile redist. H<sub>2</sub>O

up to 25 µl

Master mixes 1 and 2 were pipetted together on ice and PCR was performed as described before.

The amplified DNA fragment was purified by phenol extraction, and was recovered from an LMP gel. Following the procedure outlined in 5.3.10. the DNA fragment was ligated into the digested vector (if necessary, the vector should be dephosphorylated after restriction digestion).

### **5.4.4 DNA Sequencing**

#### **5.4.4.1. Sequencing of DNA with „ALF“ Pharmacia**

Cycle-Sequencing is based on the Dideoxy-method (Sanger et al., 1977). This method utilizes a thermostable DNA-Polymerase to synthesize a complementary copy of a single-stranded DNA template. The method makes use of the ability of the DNA polymerase and also use 2', 3'-dideoxynucleoside triphosphates (ddNTPs) as substrates. When a specific ddNTP is incorporated at the 3' end of the growing DNA chain, elongation is terminated because the chain lacks a further 3'-hydroxyl group. In each of the reactions, only one of the four possible ddNTPs is included. The ddNTP:dNTP ration in each reaction is adjusted so that chain termination occurs at each respective position of ddNTP. In this way, each of the four elongation reactions contains a population of extended chains, which have a fixed 5' end as specified by the annealed primer and a variable 3' end terminating at a specific dideoxynucleotide. The fragments can be separated by a denaturing high-resolution polyacrylamide gel according to their length. Single fragments are detected and identified by the fluorescence labeled primer.

##### **5.4.4.1.1 Preparation of sequencing gel**

The accuracy of DNA sequence determination depends largely upon resolution of the sequencing products in denaturing polyacrylamide gels. A thorough washing and rinsing of the gel plates were necessary. The part near the sample-wells was silanized with silanizing solution, wiped with ethanol and dried. The gel plates were assembled according to the manufacturer's instructions with 0.2 or 0.4 mm uniform-thickness spacers and clamps,

making sure that the side and bottom spacers fit tightly and closely together. The gel solution was filtered, then 70  $\mu$ l TEMED and 300  $\mu$ l 10 % APS were added. The gel solution was poured evenly to avoid air bubbles. Then the shark tooth comb was inserted. The gel was polymerized for at least 1.5 hr. After polymerization, the bottom spacer and the shark tooth comb were gently removed. The upper reservoir was fixed and the gel was placed in the ALF-Sequencer. The reservoirs were filled with 1 x TBE buffer.

silanizing solution:

ethanol	500 $\mu$ l
Bind Silane	2 $\mu$ l
CH <sub>3</sub> COOH, 10 %	125 $\mu$ l

gel solution:

Urea	33.6 g
10 x TBE	9.6 ml
Acrylamide/Bisacrylamide 30 % (29:1)	17.6 ml
deionized H <sub>2</sub> O	up to 80 ml

### 5.4.4.1.2 Cycle-Sequencing

For each sequencing reaction:

10 x Buffer	2.0 $\mu$ l
labelled primer (2 pmol)	1.0 $\mu$ l
dNTPs (1 mM)	4.0 $\mu$ l
Taqenase/PPase	0.16 $\mu$ l

(from Amersham Life Science, Inc. Thermosequenase; Product No.: E79000Y)

10x Puffer:

Tris-HCl, pH9.5	120 mM
MgCl <sub>2</sub>	40 mM
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	150 mM

dNTPs:

dATP, dCTP, dTTP, 7deaza-dGTP

0.5 to 1 µg DNA was added to the reaction mixture, and H<sub>2</sub>O was used to fill the volume up to 20 µl. Each 5µl of this solution was transferred into a PCR-tube, and 2 µl dideoxynucleotide solution was added to each of the four PCR tubes.

Dideoxynucleotide solution:

ddATP	5 µM
ddCTP	5 µM
ddGTP	3.75 µM
ddTTP	5 µM

PCR-reaction:

94°C	120 Sec.	1 cycle
94°C	15 Sec.	
X°C	15 Sec.	36 cycles
72°C	40 Sec.	
72°C	300 Sec.	1 cycle
4°C	Until the stop solution is added.	

„X“ equals the optimal annealing temperature of the primer.

When the PCR reaction was finished, 4 µl Stop Solution (formamide 95 %, NaOH 10 mM, bromophenol blue 0.005 %) was added to each tube. The samples were denatured at 95 °C for 2-3 min, then chilled on ice until used. The gel was run at 50°C, 1500 Volt, and 35 mA for 8 hrs. The raw data were analysed by ALF-Software.

### **5.4.4.2 Sequencing with ABI Prism 310 Genetic Analyzer**

(The chemicals are supplied by PE Applied Biosystems)

PE Applied Biosystems has developed a cycle sequencing kit with AmpliTaq DNA polymerase, FS. This enzyme is a variant of *thermus aquaticus* DNA polymerase that contains a point mutation in the active site resulting in less discrimination against dideoxynucleotides, which leads to a much more even peak intensity pattern. The second

mutation is in the amino terminal domain that virtually eliminates the 5'→3' nuclease activity of AmpliTaq DNA polymerase.

### **5.4.4.2.1 Preparing sequencing reactions**

For each reaction, the following reagents were added to a separate 0.2 ml MicroAmp PCR tube.

Terminator Ready Reaction Mix	4.0 µl
Template	400 ng
Primer	3.2 pmol
Deionized water	up to 20 µl

PCR programm :

96°C	30 sec	
X°C	15 sec	25 Cycles
60°C	4 min.	
4°C	hold	

### **5.4.4.2.2 Purification of the PCR product**

For each sequencing reaction, a 1.5 ml microcentrifuge tube was prepared containing the following: 2.0 µl of 3 M sodium acetate (pH 4.6); 50 µl 100 % ethanol. The PCR reaction was pipetted into the 1.5 ml tube and mixed thoroughly. The tube was kept on ice for 10 min to precipitate small extension products, then centrifuged 30 min at RT at maximum speed. The supernatant was carefully discarded. 250 µl 70% ethanol was used to rinse the pellet, vortexed and recentrifuged for 15 min. The pellet was dried by incubating at 37°C. 12 µl TSR (Thermosuppressor Reagent, from PE Applied Biosystems) was added and carefully mixed and spinned for 10 sec to keep the contents at the bottom. The mixture was incubated at 95°C for 2 min, then chilled on ice. After the samples had cooled they were vortexed again and spinned down. The contents of the tubes were transferred into ABI sample-vials, avoiding air bubbles. The samples were loaded in the ABI Prism 310 Genetic Analyzer before capillary electrophoresis.

Terminator Ready Reaction Mix (All chemicals are from PE Applied Biosystems) :

A-Dye Terminator labeled with Dichloro  
C-Dye Terminator labeled with Dichloro  
G-Dye Terminator labeled with Dichloro  
T-Dye Terminator labeled with Dichloro  
deoxynucleoside triphosphate (dATP, dCTP, dITP, dUTP)  
AmpliTaq DNA Polymerase, FS, with the thermally stable pyrophosphatase  
MgCl<sub>2</sub>  
Tris-HCl, pH 9.0

### **5.5 Work with eukaryotic cells**

#### **5.5.1 Cell lines**

C3H10T1/2: Fibroblast cells from mouse embryo (Reznikoff et al. 1973)  
ATCC Nr.: CCL 226  
C243/3TP-Luc: C243 cells expressing 3TP-Luc promoter construct  
NIH 3T3:

#### **5.5.2 Culture media and solutions**

DMEM (dulbecco's modified eagle's medium):

13.63 g/l DMEM powder

44 mM NaHCO<sub>3</sub>

10 mM HEPES

1 x PEN/STREP, 1 x Glutamine, and 10 % FCS are added before use

100 x PEN/STREP:

6.06 mg/ml penicillin

10 mg/ml streptomycin

100 x glutamine:

200 mM L-glutamine

FCS (fetal calf serum):

Heat-inactivated 30 min at 56°C. Aliquot and store at -20°C.

Heat-inactivated serum is required for long-term cultures. The heat treatment serves to reduce the number of viral and other adventitious contaminants and is required to inactivate complement.

PBS (phosphate buffered saline):

NaCl	150 mM
KCl	27 mM
Na <sub>2</sub> HPO <sub>4</sub>	7.2 mM
KH <sub>2</sub> PO <sub>4</sub>	14.7

TEP:

Trypsin	3 ml 2.5%
EDTA	125 µl 0.5 M , pH 7.5
PBS	100 ml

100 x Ascorbate/β-glycerophosphate:

β-glycerophosphate	9.7 g	1 M
ascorbic acid	225 mg	5mg/ml
DMEM	45 ml	

differentiation medium:

DMEM	
FCS 10 %, 1 x PEN/STREP, 1 x Glutamine, 1 x ascorbic acid/β-glycerophosphate	

G418:

100 mg/ml	stock solution
750 µg/ml	working solution

Puromycin:

2.5 mg/ml	stock solution
5 µg/ml	working solution

### **5.5.3 Culture conditions**

The adherently growing C3H10T1/2 cells were cultivated in culture flasks, dishes or 6-well-plates, 12-well-plates with DME-medium containing supplements with 5 % CO<sub>2</sub> concentration in a 37°C incubator. After reaching confluence (arbitrarily termed day 0)

differentiation medium were added as specified in the protocol of Owen *et al* (1990) for the cultivation of native osteoblast-like cells.

### **5.5.4 Conservation of eukaryotic cells**

#### **5.5.4.1 Thawing of cells**

The cells in liquid nitrogen should be rapidly unfrozen in a 37°C water bath with swirling. Then the cells were transferred into a Roux-flask (75 cm<sup>2</sup>) containing 12 ml warm medium. The medium was changed 2 hr later in order to remove DMSO contained in the freezing medium.

#### **5.5.4.2 Subcultivation**

The cells were subcultivated when they reached 80-90 % confluence. The medium was carefully removed and the cells were washed with warm PBS. Pre-warmed TEP (for a small Roux-flask 1 ml, for a middle one 2 ml) was added to the flask. After 2 or 3 min the cells became dislodged from the bottom of the flask. Pre-warmed culture medium was added to inactivate the trypsin. The cells were gently mixed and dispersed. Their number was determined with the help of a fuchs-rosenthal-counting chamber, so that the desired number of the cells could be seeded in a new flask or dish.

#### **5.5.4.3 Freezing of cells**

The cells in a medium Roux-flask were trypsinized ( 5.5.4.2), mixed with 8 ml medium, transferred to a sterile 10 ml tube, centrifuged at RT by 1500 rpm for 5 min. The supernatant was carefully sucked off, the cell pellet was resuspended in 4 ml freezing-medium (DMSO 5 %, FCS 95%). The cell suspension was aliquoted into 4 vials and kept at -70°C overnight. The next day the cells were transferred to liquid nitrogen for long-term-storage.

### **5.5.5 Fixation of cells**

#### **5.5.5.1 methanol**

After removal of culture medium, cells were washed once with PBS. The cells were covered with methanol and stored for 15 min at -20°C. Then methanol was thoroughly

socked off and the cells were washed 2 times with PBS. The cells were stored in sterile water or PBS at 4°C .

### **5.5.5.2 3 % paraformaldehyde**

Cells were washed once with PBS. 3 % paraformaldehyde was then added and incubated for 30 min at RT. After washing 2 times the cells were stored in PBS or sterile water at 4°C for several weeks.

paraformaldehyde      3 % (w/v) in PBS (heat to 80°C to dissolve, filter at RT)

### **5.5.6 Transduction of DNA into Mammalian Cells**

( **Dosper Liposomal Transfection Reagent, Boehringer Mannheim, Cat. No. 1811169**)

There are two types of transfections that are routinely performed with mammalian cells: transient and permanent ones. In a transient transfection, transcription or replication of the transfected gene can be analyzed between 1 and 4 days after introduction of the DNA. However, many experiments require formation of cell lines that contain genes that are stably integrated into chromosomal DNA resulting in a permanent transfection.

Widely used techniques include the calcium phosphate coprecipitation method (Wigler et al. 1977), electroporation (Potter et al. 1988), viral vectors (Perkus et al. 1993), and cationic liposome-mediated transfection (Felgner et al. 1987).

Dosper Liposomal Transfection reagent is a liposome preparation of the polycationic lipid 1,3-di-oleoyloxy-2-(6-carboxy-spermyl)-propylamide, consisting of a spermine head group and fatty acid tails. Due to this positively charged spermine head group, Dosper binds negatively charged DNA molecules. This binding results in the formation of polycationic complexes that are non-specifically absorbed to the overall negatively charged surface of the cells to be transfected. Subsequently, the DNA is taken up by the cell.

#### **5.5.6.1 Permanent transfection of DNA into mammalian cells**

C3H10T1/2 cells were plated into 12-well-plates at 5000 cell/cm<sup>2</sup>. The cells were incubated at 37°C in an incubator until 60-80% confluent. On the day of transfection (usually 24 hr



after plating), the culture medium was replaced by 2 ml fresh medium without serum 2 hr before transfection. The transfection mixture was prepared 1.0 hr before transfection.

Solution A: 1.5 µg DNA to be transfected and 0.15 µg selection plasmid were diluted with HBS to a final volume of 50 µl

Solution B: 6 µl Dosper was diluted with HBS to a final volume of 50 µl

Solution A and B were mixed gently by air bubbles from pipet-tips, then incubated at RT for 15 min to allow the Dosper/DNA complex to form. 900 µl of fresh medium without serum was added to the 100 µl transfection mixture and mixed gently but thoroughly. After removing the medium from 12-well-plate, this transfection medium was added onto the cells, 1 ml per well. The cells were incubated for 6 hr at 37°C. After incubation, 1 ml 20 % FCS medium was added to each well cells, so that the final concentration of serum was 10 %. 24 hr after transfection the medium was replaced by normal culture medium. The selective medium was given 48 hr after transfection. The cells were split 1:10 into new cell culture dishes, fed with selective medium every three days. The large and healthy clones were picked. RT-PCR was carried out to test whether the DNA has stably integrated into the genome of the transfected cells.

HBS:

HEPES, pH 7.4	20 mM
NaCl	150 mM
sterile-filtered	

Selection markers:

Aminoglycosidephosphotransferase (neo, G418, APH)

Selection condition: 500-800 µg/ml in complete medium

Basis for selection: G418 blocks protein synthesis in mammalian cells by interfering with ribosomal function. Expression of the bacterial APH gene in mammalian cells results in detoxification (Southern et al. 1982).

Puromycin-N-acetyltransferase (PAC, puro)

Selection condition: 5 µg/ml in complete medium

Basis for selection: Puromycin inhibits protein synthesis. The gene encodes an enzyme for the inactivation of puromycin by acetylation (de la Luna et al. 1988).

### **5.5.6.2 Transient transfection of DNA into mammalian cells**

The procedure of transient transfection was almost the same as for permanent transfection, except that solution A lacked selection plasmid and therefore after transfection the cells were fed with normal culture medium without selection marker. The cells were cultivated for appropriate time to allow expression of the transfected gene, then the cells were harvested and assayed.

### **5.5.7 RNA isolation from eukaryotic cells**

(Tri Reagent<sup>®</sup> LS, Molecular Research Center. INC.)

The TRI REAGENT<sup>®</sup>LS is a complete reagent for the isolation of total RNA or the simultaneous isolation of RNA, DNA and proteins from liquid samples of human, animal, yeast, bacterial and viral origin. It combines phenol and guanidine thiocyanate in a mono-phase solution to facilitate the immediate and most effective inhibition of RNase activity.

The medium of the cells was removed, 0.3 ml of TRI REAGENT LS per 10 cm<sup>2</sup> of culture area was added (for small flask 0.75 ml). The cells were lysed by repeatedly pipetting. Keeping the lysates at RT for 5 min permits the complete dissociation of nucleoprotein complexes. (Samples can be stored at this step at -70°C for at least one month). The lysates were transferred into an 1.5 ml eppendorf tube, supplemented with 100 µl BCP (1-bromo-3-chloropropane) per 0.75 ml of TRI REAGENT LS. The samples were mixed vigorously. The mixture was kept for 15 min at RT and then centrifuged at 12 000 x g for 15 min at 4°C. Following centrifugation, the mixture was separated into a lower red, phenol-chloroform phase, white interphase, and the colorless upper aqueous phase. RNA remains exclusively in the aqueous phase whereas DNA and proteins are in the interphase and organic phase. The volume of the aqueous phase is about 70% of the volume of TRI REAGENT LS used for homogenization. The aqueous phase was transferred to a fresh

tube carefully without disturbing the interphase. RNA was precipitated by mixing with 0.5ml isopropanol per 0.75ml of TRI REAGENT LS. Samples were kept at -70°C for 30 min. and then centrifuged at 12 000 x g for 15 min at 4 °C. RNA precipitates formed a gel-like or white pellet on the bottom of the tube. The supernatant was discarded and the RNA pellet was washed once with 1 ml 75% ethanol by vortexing and subsequent centrifugation at 12 000 x g for 5 min at 4°C. The RNA pellet was air-dried for 3-5 min and was dissolved in DEPC water. The concentration of RNA was estimated spectrophotometrically. RNA prepared by this method has an OD<sub>260/280</sub> ratio between 1.6 and 1.9.

50 - 100 µg total RNA can be isolated from a small flask. The isolated RNA was free of protein and DNA contamination and can be used for Northern analysis, dot blot hybridization, poly A<sup>+</sup> selection, in vitro translation, RNase protection assay, molecular cloning and for RT-PCR without treatment with DNase or additional purification.

### 5.5.8 Agarose/formaldehyde gel electrophoresis of RNA

1% agarose/formaldehyde gel is suitable for RNA molecules 500 bp to 10 kb in size. 1.0 g agarose was dissolved in 72 ml sterile water and cooled to 60°C in a water bath, then 10 ml of 10 x MOPS running buffer and 18 ml of 12.3 M formaldehyde were added before pouring the gel.

5 µg RNA was dried by vacuum-speed, then 2 µl DEPC H<sub>2</sub>O, 5 µl formamide, 2 µl formaldehyde, 1 µl 10 x MOPS, 1 µl EtBr (400 ng/ml), 1 µl 10 x loading buffer were added and mixed by vortexing, microcentrifuged briefly to collect the liquid, and incubated 15 min at 55°C. The gel was run at 5 V/cm in 1 x MOPS running buffer until the bromophenol blue dye had migrated one-half to two-thirds the length of the gel.

10 x MOPS:

MOPS	200 mM
EDTA	10 mM
NaAc, pH 7.1	10 mM

10 x Loading Buffer:

bromophenol blue	0.25 % (w/v)
------------------	--------------

xylene cyanol	0.25 % (w/v)
glycerol	50 % (w/v)
EDTA, pH 8.0	1 mM

**5.5.9 Histological methods****5.5.9.1 Alkaline phosphatase staining (Osteoblast staining)**

(SIGMA FAST™ BCIP/NBT Buffered Substrate Tablet, Cat. No. B-5655)

Osteoblasts exhibit a stellate morphology displaying high levels of alkaline phosphatase activity which was visualized by cellular staining with SIGMA FAST BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) tablets. The tablets have been developed as a precipitating substrate for the detection of alkaline phosphatase activity. One tablet is dissolved in 10 ml of deionized and distilled water. The solution contains:

BCIP (5-bromo-4-chloro-3-indolyl phosphate)	0.15 mg/ml
NBT (nitro blue tetrazolium) chloride	0.30 mg/ml
Tris buffer	100 mM
MgCl <sub>2</sub>	5 mM

Alkaline Phosphatase + BCIP → BCI + PO<sub>4</sub>, pH 9.5

BCI + NBT → insoluble, blue color

For the fixed cells in 6-well-plate, 800 µl substrate solution was added per well. The 6-well-plate was kept in the dark for 1 hr. Then the solution was soaked off, and the cells were washed twice with PBS. The cells were stored in PBS or H<sub>2</sub>O at 4°C.

**5.5.9.2 Alcian Blue staining (Chondrocyte staining)**

The fixed cells were washed with PBS, then covered with alcian blue solution (0.5 % alcian blue in 1 N HCl). After 6 hr to overnight incubation at RT, the alcian blue solution was soaked out. The cells were washed twice with PBS and stored in PBS or H<sub>2</sub>O at 4°C. The chondrocytes are identified by displaying blue color, whereas the other cells are colorless.

**5.5.10 Reporter gene assays**

### 5.5.10.1 Overview of genetic reporter systems

A central question in molecular and cell biology is how *cis-acting* DNA sequences and *trans-acting* factors act in union to control eukaryotic gene expression. These interactions are mediated by specific binding of a transcription factor or a complex of factors to enhancer and promoter elements generally found upstream of the transcription start site. Direct quantitation of changes in gene expression requires the measurement of specific mRNAs using techniques such as northern blot hybridizations or nuclease protection assays. But these procedures can be time-consuming and are not always adequate for analysis of many different gene constructs and require that the analyzed gene be modified in some manner to distinguish it from the native gene in transfected cells.

An alternative approach to monitor changes in transcription is to link the presumed *cis-acting* sequences from the gene of interest to the coding sequence for an unrelated reporter gene (Alam and Cook, 1990). To test for complete promoters, the DNA fragment is placed directly upstream of the reporter gene in a vector lacking endogenous promoter activity. After introduction of the reporter constructs into an appropriate cell type, measurement of reporter-gene products provides an indirect estimate of the induction in gene expression directed by the regulatory sequences. The reporter assays thus take place at the protein level but not from RNA level. The two levels are frequently, but not always, correlated with one another.

### 5.5.10.2 $\beta$ -galactosidase reporter assay

#### $\beta$ -Gal Reporter Gene Assay Kit (chemiluminescent), Boehringer Mannheim

The *LacZ* gene from *E. coli*, which encodes the enzyme  $\beta$ -galactosidase, is among the most versatile genetic reporters, having a variety of different substrates. The enzyme catalyzes the hydrolysis of various  $\beta$ -galactosides. In addition to its use as a reporter for uncharacterized *cis*-regulatory sequences, expression of  $\beta$ -galactosidase under the control of a constitutive promoter is frequently used as an internal control to normalize the variability of other reporter assays.  $\beta$ -galactosidase is particularly useful for normalizing CAT and firefly luciferase.

This assay has two basic steps:

### **1. Enzyme reaction:**

In this step the substrate, Galacton Plus™ (supplied by  $\beta$ -Gal Reporter Gene Assay Kit), becomes deglycosylated by the enzymatic activity of the  $\beta$ -galactosidase contained in the sample. This incubation step was performed at pH 7.8, where bacterial  $\beta$ -galactosidase is highly active. At this neutral pH, the cleaved dioxetane is protonated and will not produce a light signal. This stable intermediate accumulates during the reaction, and provides a light signal once the pH will be raised.

### **2. Light reaction:**

The light reaction is initiated by shifting the pH to a value above 12. Due to this shift in pH the activated intermediate becomes deprotonated and decomposes with the emission of light (475 nm). The presence of special polymeric enhancer substances improves the quantum yield of the chemiluminescent reaction. The light signal is measured by a luminometer.

The  $\beta$ -galactosidase expression plasmid-transfected cells were washed three times with cold PBS. After careful removal of PBS, 300  $\mu$ l of 1 x Cell Lysis Buffer was added per well of a 12-well-plate. The cells were allowed to incubate for 30 min at RT. All soluble cytoplasmic and nucleoplasmic components, including  $\beta$ -galactosidase, were extracted by the lysis buffer. In the case of adherent cells, nuclei (including DNA packaged in chromatin) will remain attached to the vessel surface (Lu et al., 1992).

Cell extracts were transferred into a 1.5 ml eppendorf tube, centrifuged for 5 min. at maximum speed at 4°C to precipitate cellular debris. The supernatant was then transferred into a new tube, cell extracts were used immediately or should be stored frozen at -70°C.

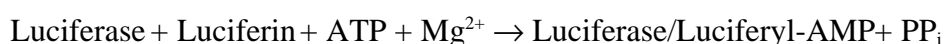
Depending on the cell line used, substantial amounts of endogenous lysosomal  $\beta$ -galactosidase may be present in the lysate and contribute to the non-specific background. To reduce background, cell lysate was incubated for 60 min at 50°C (Young et al., 1993).

30  $\mu$ l deionized, sterile water and 20  $\mu$ l cell extracts were added per well of a MTP (microtiter plate, LB 96-WMP, BERTHOLD). After adding 100  $\mu$ l Substrate Reagent, the MTP was covered with a cover foil to avoid evaporation, and was incubated 60 min at RT, while rocking gently. The Initiation Reagent (1 vol. of Enhancer + 5 vol. Initiation solution) was diluted with redist. water so that the amount of the Initiation Reagent be one fourth of the final volume. The MTP was placed into the luminometer (Microlumet LB 96, product

from firm EG & G Berthold), 100 µl Initiation Reagent was automatically injected. After a delay of 2 sec, start integration of light production for 8 sec.

### 5.5.10.3 Firefly luciferase reporter assay

Cloning of the *luc* gene from the firefly *Photinus pyralis* (de Wet et al. 1987) provided the first nonisotopic genetic reporter system with widespread utility in mammalian cells. The bioluminescent reaction catalyzed by luciferase requires luciferin (the substrate), ATP,  $Mg^{2+}$ , and molecular  $O_2$ . Mixing these reagents with cell lysates containing luciferase results in a flash of light that decays rapidly. The light signals are detected by the luminometer (Lumat LB 9501, product from Berthold). The total light emission is proportional to the luciferase activity of the sample, which in turn provides an indirect estimate of the transcription of the luciferase reporter gene. Luciferase is sensitive to degradation by proteases, and therefore has a half-life of about 3 hrs in transfected mammalian cells (Thompson et al. 1993). The following reactions are included:



The transfected cells were washed twice with cold PBS to remove the calcium containing medium thoroughly so that the luciferase reaction is not inhibited by traces of calcium. 1 x cell lysis buffer from the  $\beta$ -Galactosidase Kit (Boehringer) was used to lyse the cells. The procedure is as described in 5.4.10.2.

Cell lysates were gently mixed. 20 µl of the cell lysate was added into 350 µl Luciferase Reaction Buffer in a measuring tube and mixed gently. The measuring tube was inserted into the luminometer chamber and was immediately measured. The injector of the counter was pre-rinsed with diluted luciferin. 50 µl diluted luciferin solution was injected per sample in the luminometer and light output was measured for 10 sec at 25°C.

Reaction Buffer:

glycylglycine, pH 7.8	25 mM
MgSO <sub>4</sub>	15 mM
ATP	5 mM
pH is adjusted with KOH to 7.8	

Normally the reaction buffer is made up as 1.25 x stock solution without ATP. Before use, mix 10 ml 1.25 x stock solution with 2.5 ml 25 mM ATP.

luciferin solution:

luciferin	0.4 mM
glycylglycine,pH 7.8	25 mM

luciferin stock solution: 10 mM

luciferin 10 mg in 3.57 ml glycylglycine. The stock solution was divided into 40 µl aliquots and stored in the dark at -20°C. When needed, 1 ml glycylglycine (pH 7.8) was added into the tube.

### 5.5.10.4 Statistic evaluation

The luciferase activities were normalized on the basis of β-galactosidase expression from pSV β-galactosidase in all luciferase reporter experiments. All the experiments were repeated at least three times with similar results. The mean and standard error were calculated by the following formula:

$$\bar{X} = \frac{\sum_{i=1}^n X_i}{n} \quad S_x = \sqrt{\frac{\sum X_i^2 - n\bar{X}^2}{n - 1}}$$

$\bar{X}$                       mean

$S_x$                       standard error

$n$                         the number of samples

$X_i$                       counts of a sample



**6 References**

**Ahrens, M., Ankenbauer, T., Schröder, D., Hollnagel, A., Mayer, H., and Gross, G.:**

Expression of Human Bone Morphogenetic Proteins-2 or -4 in Murine Mesenchymal Progenitor C3H10T1/2 Cells induces Differentiation into Distinct Mesenchymal Cell Lineages. *DNA cell Biol.* 12 (1993), 871-880.

**Alam, J. and Cook, J.L:** Reporter genes: Application to the study of mammalian gene transcription. *Anal. Biochem.* 188 (1990), 245-254.

**Angel, P, and Karin, M.:** The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim. Biophys. Acta* 1072 (1991), 128-157.

**Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R.J., Rahmsdorf, H.J., Jonat, C., Herrlich, P., and Karin, M.:**

Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. *Cell* 49 (1987), 729-739.

**Arora, K., Dai, H., Kazuko, S.G., Jamal, J., O'conor, M.B., Letson, A., and Warrior, R.,:**

The *Drosophila schnurri* gene acts in the Dpp/TGF- $\beta$  signalling pathway and encodes a transcription factor homologous to the human BMP family. *Cell* 81 (1995), 781-790.

**Attisano, L., and Wrana, J.L.:** Signal transduction by members of the transforming growth factor- $\beta$  superfamily. *Cytokine Growth Factor Rev* 7 (1996), 327-339.

**Baker, J. and Harland, R.M. :** A novel mesoderm inducer, *Madr2*, functions in the activin signal transduction pathway. *Genes Dev.* 10 (1996), 1880-1889.

**Baker, J.C. and Harland, R.M.:** From receptors to nucleus: the Smad pathway. *Current Opinion in Genetics & Development* (1997), 7: 467-473.

**Basilico, C., and Moscatelli, D.:** The FGF family of growth factors and oncogenes. *Adv. Canc. Res.* 59 (1992), 115-165.

**Birnboim, H.C., Doly, J.:** A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* (1979), 7, 1513-1523.

**Bitgood, M.J., and McMahon, A.P.:** Hedgehog and BMP genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. *Dev. Biol.* 172 (1995), 126-138.

**Brenner, C. A., Tam, A. W., Nelson, P. A., Engleman, E. G., Suzuki, N., Fry, K. E., and Larrick, J. W.:**

Message Amplification Phenotyping (MAPPING): a technique to simultaneously measure multiple mRNAs from small numbers of cells. *BioTechniques* 7 (1989), 1096-1103.

**Carlson, C.S., Tulli, H.M., Jayo, M.J., Loeser, R.F., Tracy, R.P., Mann, K.B., and Adams, M.R.:**

Immunolocalization of noncollagenous bone matrix proteins in lumbar vertebrae from intact and surgically menopausal cynomolgus monkeys. *J. Bone. Min. Res.* 8 (1993), 71-81.

**Celeste, A.J., Iannazzi, J.A., Taylor, R.C., Hewick, R.M., Rosen, V., Wang, E.A., and Wozney, J.M.:**

Identification of transforming growth factor beta family members present in bone-inductive protein purified from bovine bone. *Proc. Natl. Acad. Sci.* 87 (1990), 9843-9847.

**Chaloux, E., López-Rovira, T., Rosa, J.L., Bartons, R., and Ventura, F.:** JunB is involved in the inhibition of myogenic differentiation by bone morphogenetic protein-2. *J.B.C.* 273 (1998), 537-543.

- Chattopadhyay, N., Mithal, A., and Brown, E.M.:** The calcium-sensing receptor: a window into the physiology and pathophysiology of mineral ion metabolism. *Endocr. Rev.* 17 (1996), 289-307.
- Chelly, J., Kaplan, J.-C., Gautron, S. and Kahn, A.:** Transcription of the dystrophin gene in human muscle and non-muscle tissues. *Nature* 333 (1988), 858-860.
- Chen, T.L., Bates, R.L., Dudley, A.S., Hammonds, Jr.R.G., and Amento, E.P.:** Bone morphogenetic protein-2b stimulation of growth and osteogenic phenotypes in rat osteoblast-like cells, comparison with TGF- $\beta$ 1. *J. Bone. Min. Res.* 6 (1991), 1387-1393.
- Chen, X., Rubock, M.J., and Whitman, M.:** A Transcriptional partner for MAD proteins in TGF- $\beta$  signalling. *Nature* 383 (1996), 691-696.
- Chen, X., Weisberg, E., Fridmacher, V., Watanabe, M., Naco, G., and Whitman, M.:** Smad4 and FAST-1 in the assembly of activin-responsive factor. *Nature* 389 (1997), 85-89.
- Chen, Y., Lebrun, J.-J., and Vale, W.:**  
Regulation of transforming growth factor- $\beta$ - and activin-induced transcription by mammalian Mad proteins. *Proc. Natl. Acad. Sci. USA.* 93 (1996), 12992-12997.
- Childs, S.R., Wrana, J.L., Arora, K. et al:** Identification of a *Drosophila* activin receptor. *Proc. Natl. Acad. Sci. USA* 90 (1993), 9475-9479.
- Chiu, R., Angel, P., and Karin, M.:** JunB differs in its biological properties from, and is a negative regulator of, c-Jun. *Cell* 59 (1989), 979-986.

- Colbe're-Garapin, F., Horodiniceanu, F., Kourisky, P., and Garapin, A.C.:** A New Dominant Hybrid Selective Marker for Higher Eukaryotic Cells. *J. Mol. Biol.* 150 (1981), 1
- Das, P., Maduzia, L.L., Wang, H., Finelli, A.L., Cho, S-H., Smith, M.M., and Padgett, R.W.:**  
The *Drosophila* gene *Medea* demonstrate the requirement for different classes of Smads in *dpp* signaling. *Development* 125 (1998), 1519-1528.
- Davidson, D.:** The function and evolution of *MSX* genes: pointers and paradoxes. *Trends Genet.* 11 (1995), 405-411.
- De Caestecker, M.P., Hemmati, P., Larisch-Bloch, S., Ajmera, R., Roberts, A., and Lechleider, R.:**  
Characterization of functional domains within Smad4/DPC4. *J. B.C.* 272 (1997), 13690-13696.
- De Groot, R.P., and kruijer, W.:** Transcriptional activation by TGF $\beta$ , mediated by the dyad symmetry element (DSE) and the TPA responsive element (TRE). *Biochem. Biophys. Res. Commun.* 168 (1991), 1074-1081.
- De LA LUNA, S.:** Efficient transformation of mammalian cells with constructs containing a puromycin-resistance marker. *Gene* 62 (1988), 121-126.
- Deng, C., Wynshaw-Boris, A., Zhou, F., Kuo, A., and Leder, P.:** Fibroblast growth factor receptor 3 is a negative regulator of bone growth. *Cell* 84 (1996), 911-921.
- Dennler, S., Itoh, S., Vivien, D., ten Dijke, P., Huet, S. and Gauthier, J-M.:** Direct binding of Smad3 and Smad4 to critical TGF $\beta$ -inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *EMBO* 17 (1998), 3091-3100.

**Derynck, R., Gelbart, W.M., Harland, R.M., Heldin, C-H., Kern, S.E., Massague', J., Melton, D.A., Mlodzik, M., Padgett, R.W., Roberts, A.B., Smith, J., Thomsen, G.H., Vogelstein, B., and Wang, X-F.:**

Nomenclature: Vertebrate mediators of TGF- $\beta$  Family Signals. Cell 87 (1996a), 173.

**Derynck, R., and Feng, X-H.:** TGF- $\beta$  receptor signaling. Biochimica et Biophysica Acta 1333 (1997), F105-F150.

**Derynck, R., and Zhang, Y.:** Intracellular signalling: The Mad way to do it. Current Biology 6 (1996b), 1226-1229.

**De Wet, J.R., Wood, K.V., Deluka, M., Helinski, D.R., and Subramani, S.:** Firefly luciferase gene: Structure and expression in mammalian cells. Mol. Cell. Biol. 7 (1987), 725-737.

**Doctor, J.S., Jackson, P.D., Rashka, K.E., Visali, M., and Hoffmann, F.M.:** Sequence, biochemical characterization, and developmental expression of a new member of the TGF-beta superfamily in *Drosophila melanogaster*. Dev. Biol. 151 (1992), 491-505.

**Dower, W. J. Miller, J.F., and Ragadale, C.W.:** High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. 16 (1988), 6127-6145.

**Ducy, P., Zhang, R., Geoffroy, V., Ridall, A.L., and Karsenty, G.:** Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 89 (1997), 747-754.

**Eppert, K., Scherer, S.W., Ozcelik, H., Pirone, R., Hoodless, P., Kim, H., Tsui, L-C., Bapat, B., Gallinger, S., Andrulis, I.L., Thomsen, G.H., Wrana, J.L., and Attisano, L.:** Madr2 maps to 18q21 and encodes a TGF- $\beta$  -regulated Mad-related protein that is functionally mutated in colorectal carcinoma. Cell 86 (1996), 543-552.

- Erlebacher, A., Filvaroff, E.H., Gitelman, S., and Derynck, R.:** Toward a molecular understanding of skeletal development. *Cell* 80 (1995), 371-378.
- Felgner, J. et al.:** *J. Tiss. Cult.Meth.* 15(1993), 63.
- Frohman, M.A.:** Rapid amplification of cDNA ENds (RACE): User-Friendly cDNA Cloning. *Amplification* 5 (1990), 11-15.
- Gitelman, S.E., Kobrin, M.S., Ye, J-Q., et al.:** Recombinant Vgr-1/BMP6-expressing tumors induce fibrosis and endochondral bone formation in vivo. *J. Cell. Biol.* 126 (1994), 1595-1609.
- Gimble, J.M., Morgan, C., Kelly, K., Wu, X., Dandapani, V., Wang, C-S., and Rosen, V.:**  
Bone morphogenetic proteins inhibit adipocyte differentiation by bone marrow stromal cells. *J. Cell. Biochem.* 58 (1995), 393-402.
- Graff, J.M., Bansal, A., and Melton, D.A.:** *Xenopus* Mad proteins transduce distinct subsets of signals of the TGF- $\beta$  superfamily. *Cell* 85 (1996), 479-487.
- Grieder, NC., Nellen, D., Burke, R., Basler, K., and Affolter, M.:** Schnurri is required for *Drosophila* Dpp signalling and encodes a zinc finger protein similar to the mammalian transcription factor PRD II-BF1. *Cell* 81(1995), 791-800.
- Hashimoto, M., Gaddy-kurten, D., and Vale, W.:** Protooncogene junB as a target for activin actions. *Endocrinology* 133 (1993), 1934-1940.
- Hata, A., Lagna, G., Massagué, J., and Hemmati-Brivanlou, A.:** Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor. *Genes Dev.* 12 (1998), 186-197.

- Hata, A., Lo, R.S., Wotton, D., Lagna, G., Massagué:** Mutations increasing autoinhibition inactivate tumour suppressors Smad2 and Smad4. *Nature* 388 (1997), 82-87.
- Hattersley, G., Hewick, R., and Rosen, V.:** In situ localization and in vitro activity of BMP-13. *J. Bone. Miner. Res.* 10 (1995), S 163.
- Hauschka, P.V.:** Osteocalcin: the Vitamin K-dependent Ca<sup>2+</sup>-binding protein of bone matrix. *Haemostasis* 16 (1986), 258-272.
- Hauschka, P.V., and Gallop, P.M.:** Purification and calcium-binding properties of osteocalcin, the gamma-carboxyglutamate-containing protein of bone; in Wasserman, Calcium binding proteins and calcium function, pp. 338-347. (Elsevier/North-Holland, Amsterdam 1977).
- Hayashi, H. Abdollah, S., Qiu, Y., Cai, J.X., Xu, Y-Y., Grinnell, B.W., Richardson, M.A., Topper, J.N., Gimbrone, M.A., Wrana, J.L., and Falb, D.:**  
The MAD-related protein Smad 7 associates with the TGF $\beta$  receptor and functions as an antagonist of TGF $\beta$  signalling. *Cell* 89 (1997), 1165-1173.
- Heersche, J.N.M., Reimers, S., Wrana, J.L., Waye, M.M.Y., and Gupta, A.K.:**  
Changes in expression of alpha 1 type I collagen and osteocalcin mRNA in osteoblasts and odontoblasts at different stages of maturity as shown by *in situ* hybridization. *Proc. Finn. Dent. Soc.* 88 (Suppl.1. 1992), 173-182.
- Helder, M.N., Bronckers, A.L., and Woltgens, J.H.:** Dissimilar expression patterns for the extracellular matrix proteins osteopontin (OPN) and collagen I in dental tissues and alveolar bone of the neonatal rat. *Matrix* 13 (1993), 415-425.
- Heldin, C-H., Miyazono, K., and ten Dijke, P.:** TGF- $\beta$  signaling from cell membrane to nucleus through SMAD proteins. *Nature* 390 (1997), 465-471.

- Hiraki, Y. et al.:** Bone morphogenetic proteins ( BMP-2 and BMP-3) promote growth and expression of the differentiated phenotype of rabbit chondrocytes and osteoblastic MC3T3-E1 cells *in vitro*. J. Bone. Min. Res. 6 (1991), 1373-1385.
- Hogan, B.L.M.:** Bone morphogenetic proteins: multifunctional regulators of vertebrate development. Genes and Development 10 (1996a), 1580-1594.
- Hogan, B.L.M.:** Bone morphogenetic proteins in development. Curr. Opin. Genet. Dev. 6 (1996b), 432-438.
- Hogan, B.L.M., Blessing, M., Winnier, G.E., Suzuki, N., and Jones, C.M.:** Growth factors in development: the role of TGF- $\beta$  related polypeptide signalling molecules in embryogenesis. Development 1994 supplement, 53-60.
- Hollnagel, A., Ahrens, M., and Gross, G.:** Parathyroid hormone enhances early and suppresses late stages of osteogenic and chondrogenic development in a BMP-dependent mesenchymal differentiation system (C3H10T1/2 ). Journal of Bone and Mineral Research 12 (1997), 1993-2004.
- Hoodless, P.A., Haerry, T., Abdollah, S., Stapleton, M., O'Connor, M.B., Attisano, L., and Wrana, J.L.:**  
Madr1, a MAD-related protein that functions in BMP2 signalling pathways. Cell 85 (1996), 489-500.
- Hoodless, P.A., And Wrana, J.L.:** Mechanism and Function of Signaling by the TGF- $\beta$  Superfamily. Protein molecules in signal Transduction. Series: Current topics in Micro- Biology and Immunology. 228 (1998), 235-272.
- Hu, E., Liang, P., and Spiegelman, B.M.:** AdipoQ is a novel adipose-specific gene dysregulated in obesity. J. Bio. Chem. 271 (1996), 10697-10703.



- Hu, P.P-C., Datto, M.B., and Wang, X-F.:** Molecular Mechanisms of Transforming Growth Factor- $\beta$  Signaling. *Endocrine Reviews* 19 (1998), 349-363.
- Hudson, J.B., Podos, S.D., Keith, K., Simpson, S.L., and Ferguson, E.L.:** The *Drosophila Medea* gene is required downstream of *dpp* and encodes a functional homolog of human Smad4. *Development* 125 (1998), 1407-1420.
- Imamura, T., Takase, M., Nishihara, A., Oeda, E., Hanai, J-I., Kawabata, M., and Miyazono, K.:**  
Smad6 inhibits signalling by the TGF- $\beta$  superfamily. *Nature* 389 (1997), 622-626.
- Inoue, H., Imamura, T., Ishidou, Y., Takase, M., Udagawa, Y., Oka, Y., Tsuneizumi, K., Tabata, T., Miyazono, K., and Kawabata, M.:**  
Interplay of signal mediators of Decapentaplegic (Dpp): molecular characterization of Mothers against dpp, Medea, and Daughters against dpp. *Mol. Biol. Cell.* 9 (1998), 2145-2156.
- Ito, Y.:** Structural alterations in the transcription factors PEBP2/CBF linked to four different types of leukemia. *J. Cancer Res. Clin. Oncol.* 122 (1996), 266-274.
- Jeffery, J.J., and Martin, G.R.:**  
The role of ascorbic acid in the biosynthesis of collagen. *Biochim. Biophys. Acta* 121 (1966), 269-280.
- Johnson, R.S., Spiegelman, B.M., and Papaioannou, V.:** Pleiotropic effects of a null mutation in the c-fos proto-oncogene. *Cell* 71 (1992), 577-586.
- Jonk, L.J.C., Itoh, S., Heldin, C-H., ten Dijke, P., and Kruijer, W.:** Identification and functional characterization of Smad binding element (SBE) in the JunB promoter that acts as a transforming growth factor- $\beta$ , activin, and bone morphogenetic protein-inducible enhancer. *J. Bio. Chem.* 273 (1998), 21145-21152.

**Jüppner, H., Abou-Samra, A-B., Freemann, M., Kong, XF., Schipani, E., Richards, J., Kolakowski, LFJ., Hock, J., Potts, JRJ., Kronenberg, HM., and Segre, GV.:**

A G protein -linked receptor for parathyroid hormone and parathyroid hormone-related peptide. *Science* 254 (1991), 1024-1026.

**Kania, M.A., Bonner, A.S., Duffy, J.B., and Gergen, J.P.:** The *Drosophila*

segmentation gene *runt* encodes a novel nuclear regulation protein that is also expressed in the developing nervous system. *Genes Dev.* 4 (1990), 1701-1703.

**Kawasaki, E. S.:** Amplification of RNA. In: *PCR Protocols, A Guide to methods and Applications* (1991), Innis *et al.*, eds., Academic Press, Inc., San Diego, CA, pp. 21-27.

**Kim, J. Johnson, K., Chen, H.J., Carroll, S., and Laughon, A.:** *Drosophila Mad* binds to DNA and directly mediates activation of *vestigal* by Decapentaplegic. *Nature* 388 (1997), 304-308.

**Kingsley, D.M.:** The TGF- $\beta$  superfamily: New members, new receptors, and new genetic tests of function in different organisms. *Genes and Development* 8 (1994), 133-146.

**Komori, T., and Kishimoto, T.:** *cbfa1* in bone development. *Current Opinion in Genetics and Development* 8 (1998), 494-499.

**Kretzschmar, M., and Massagué, J.:** SMADs: mediators and regulators of TGF- $\beta$  signaling. *Current Opinion in Genetics and Development* 8 (1998), 103-111.

**Kretzschmar, M., Doody, J., and Massagué, J.:** Opposing BMP and EGF signaling pathways converge on the TGF- $\beta$  family mediator Smad1. *Nature* 389 (1997), 618-622.

- Kretzschmar, M. Liu, F., Hata, A., Doody, J., and Massagué, J.:** The TGF- $\beta$  family mediator Smad1 is phosphorylated directly and activated functionally by the BMP receptor kinase. *Genes & Development* 11(1997a), 984-995.
- Kronenberg, H.M.:** Editorial: Parathyroid Hormone and Osteocalcin-when friends become strangers. *Endocrinology* 138 (1997), 3083-3084.
- Kronenberg, H.M., Abou-Samra, A-B., Bringhurst, F.R., Gardella, T.J., Jüppner, H., and Segre, G.V.:**  
The PTH/PTHrP receptor: one receptor for two ligands. In: Thakker RV(ed) *Molecular Genetics of Endocrine Disorders*. Chapman & Hall, London, 1997, pp 389-420.
- Lagna, G., Hata, A., Hemmati-Brivanlou, A., and Massagué, J.:** Partnership between DPC-4 and SMAD proteins in TGF- $\beta$  signalling pathways. *Nature* 383 (1996), 832-836.
- Lauber, J.:** Genetische Charakterisierung der östrogenabhängigen Entwicklung von Knochenmarksstromazellender Ratte in vitro. Dissertation (1998), Technische Universität Braunschweig
- Lechleider, R.J., de Caestecker, M.P., Dehejia, A., Polymeropoulos, M.H., and Roberts, M.B.:**  
Serine phosphorylation chromosomal localization and TGF- $\beta$  signal transduction by human bsp-1. *J. Biol. Chem.* 271 (1996), 17617-17620.
- Li, L., Hu, J.S., and Olson, E.N.:** Different members of the jun proto-oncogene family exhibit distinct patterns of expression in response to type beta transforming growth factor. *J. Biol. Chem.* 265 (1990), 1556-1562.

**Lin, H.Y., Wang, X-F., Ng-Eaton, E., Weinberg, R.A., and Lodish, H.F.:**Expression  
Cloning of the TGF- $\beta$  Type II Receptor, a Functional Transmembrane  
Serine/threonine Kinase Cell 68 (1992), 775-785.

**Lin, L.F.H., Doherty, D.H., Lile, J.D., Bektesh, S., and Collins, F.:**  
GDNF: a glial cell line-derived neurotropic factor for midbrain dopaminergic  
neurons. Science 260 (1993), 1130-1132.

**Lipman, M.L., Panda, D., Bennett, H.P.J., Henderson, J.E., Shane, E., Shen, Y.N.,  
Goltzman, D., and Karaplis, A.C.:**  
Cloning of Human *PEX* cDNA. J. Bio. Chem. 273 (1998), 13729-13737.

**Liu, F., Hata, A., Baker, J.C., Doody, J., Cácamo, J., Harland, R.m., and Massagué,  
J.:**  
A human Mad protein acting as a BMP-regulated transcriptional activator. Nature  
381 (1996), 620-623.

**Liu, F., Pouponnot, C., and Massagué, J.:**  
Dual role of the Smad4/DPC4 tumor suppressor in TGF $\beta$ -inducible transcriptional  
complexes. Genes Dev. 11 (1997), 3157-3167.

**Lu, J. and Jiang, C.:** Biothchniques 12 (1992), 643.

**Lyons, K.L., Graycar, J.L., Lee, A., Hashmi, S., Lindquist, P.B., Chen, E.Y., Hogan,  
B.L., and Derynck, R.:**  
Vgr-1, a mammalian gene related to *Xenopus* Vg-1, is a member of the  
transforming growth factor beta gene superfamily. Proc. Natl. Acad. Sci. 86  
(1989), 4554-4558.

**Macias-Silva, M. Abdollah, S., Hoodless, P.A., Pirone, R., Attisano, L., and Wrana,  
J.L.:**

MADR2 is a substrate of the TGF- $\beta$  receptor and its phosphorylation is required for nuclear accumulation and signaling. *Cell* 87 (1996), 1215-1224.

**Maniatis, R., Fritsch, E.F., Sambrook, J.:** in 'Molecular cloning'; 1989, Cold Spring Harbor Laboratory Press.

**Mark, M.P., Prince, C.W., Gay, S., Austin, R.L., Bhowm, M., Finkelman, R.D., and Butler, W.T.:**

A comparative immunocytochemical study on the subcellular distribution of 44 kDa phosphoprotein and bone gamma-carboxyglutamic acid (Gla)-containing protein in osteoclasts. *J. Bone. Min. Res.* 2 (1987), 337-346.

**Massagué, J.:** The transforming growth factor- $\beta$  family. *Annu. Rev. Cell. Biol.* 6 (1990), 597-641.

**Massagué, J.:** TGF- $\beta$  signaling: receptors, transducers and MAD proteins. *Cell* 85 (1996), 947-950.

**Massagué, J., Hata, A., and Liu, F.:** TGF- $\beta$  signalling through the Smad pathway. *Trends in Cell Biology* 7 (1997a), 187-192.

**Massagué, J., and Weis-Garcia, F.:** Serine/threonine kinase receptors : Mediators of transforming growth factor beta family signals. In: Pawson T, Parker P ( eds) *Cancer surveys cell signaling*. Vol 27. ICRF Press, London, 1996, pp41-64.

**Meno, C., Saijoh, Y., Fuji, H., Ikeda, M., Yokoyama, T., Yokoyama, M., Toyoda, Y., and Hamada, H.:**

Left-right asymmetric expression of the TGF $\beta$ -family member lefty in mouse embryos. *Nature* 381 (1996): 151-155.

**Meersseman, G. Verschueren, K., Nelles, L., Blumenstock, C., Kraft, H., Wuytens, G., Remacle, J., Kozak, C.A., Tylzanowski, P., Niehrs, C., and Huylebroeck, D.:**

- The C-terminal domain of Mad-like signal transducers is sufficient for biological activity in the *Xenopus* embryo and transcriptional activation. *Mechanisms of Development* 61 (1997), 127-140.
- Miyazono, K., ten Dijke, P., Ichijo, H., and Heldin, C-H.:** Receptors for transforming growth factor- $\beta$ . *Adv. Immunol* 55 (1994), 181-220.
- Nakao, A., Afrakhte, M., Morén, A., Nakayama, T., Christian, J.L., Heuchel, R., Itoh, S., Kawabata, M., Heldin, N-E., Heldin, C-H., and ten Dijke, P.:** Identification of Smad7, a TGF $\beta$ -inducible antagonist of TGF- $\beta$  signaling. *Nature* 389 (1997), 631-635.
- Nakao, A., Imamura, T., Souchelnytskyi, S., Kawabata, M., Ishisaki, A., et al.:** TGF- $\beta$  receptor-mediated signalling through Smad2, Smad3 and Smad4. *EMBO J.* 16 (1997), 5353-5362.
- Newfeld, S.J., Chartoff, E.H., Graff, J.M., Melton, D.A., and Gelbart, W.M.:** Mothers against *dpp* encodes a conserved cytoplasmic protein required in DPP/TGF- $\beta$  responsive cells. *Development* 122 (1996), 2099-2108.
- Otto, F., Thornell, A.P., Crompton, T., Denzel, A., Gilmour, K.C., Rosenwell, I.R., Stamp, G.W.H., Beddington, R.S.P., Mundlos, S., Olsen, B.R., Selby, P.B., and Owen, M.J.:** *cbfa* 1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell* 89 (1997), 765-771.
- Owen, T. A., Aronow, M., Shalhoub, V., Barone, L. M., Wilming, L., Tassinari, M.S., Kennedy, M.B., Pockwinse, S., Lian, J. B., and Stein, G. S.:** Progressive development of the rat osteoblast phenotype In Vitro: reciprocal relations in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. *J. Cell. Physiol.* 143 (1990), 420-430.

**Ozkaynak, E., Rueger, D.C., Drier, E.A., Corbett, C., Ridge, R.J., Sampath, T.K., and Oppermann, H.:**

OP-1 cDNA encodes an osteogenic protein in the TGF-beta family. EMBO J. 9 (1990), 2085-2093.

**Ozkaynak, E., Schnegelsberg, P.N., Jin, D.F., Clifford, G.M., Warren, F.D., Drier, E.A., and Oppermann, H.:**

Osteogenic protein-2. A new member of the transforming growth factor-beta superfamily expressed early in embryogenesis. J. Biol. Chem. 267 (1992), 25220-25227.

**Padgett, R.W., Das, P., and Krishna, S.:**

TGF- $\beta$  signaling, Smads, and tumor suppressors. Bioessays 20 (1998a), 382-391.

**Padgett, R.W., Cho, S-H., and Evangelista, C.:**

Smads are the central component in the transforming growth factor- $\beta$  signaling. Pharmacol. Ther. 78 (1998), 47-52.

**Padgett, R.W., St. Johnston, R.D., and Gelbart, W.M.:** A transcript from a *Drosophila* pattern gene predicts a protein homologous to the transforming growth factor- $\beta$  family. Nature 325 (1987), 81-84.

**Raftery, L.A., Twombly, V., Wharton, K., and Gelbart, W.M.:** Genetic screens to identify elements of the decapentaplegic signaling pathway in *Drosophila*. Genetics 139 (1995), 241-254.

**Rappolee, D. A., Mark, D., Banda, M. J., and Werb, Z.:** Wound macrophages express TGF- $\alpha$  and other growth factors in vivo: analysis by mRNA phenotyping. Science 241 (1991), 708-712.

**Rasmussen, H., and Tenenhouse, H.S.:** Hypophosphatemias in ‘ The Metabolic Basis of inherited Diseases’ ( Scriver, C.R., Beaudet, A.L., Sly, W.S., and Valle, D. Eds.) 6th ed., 1989, pp. 2581-2604. McGraw-Hill, New York.

**Reimold, A.M., Grusby, M.J., Kosaras, B., Fries, J.W., Mori, R., Maniwa, S., Clauss, I.M., Collins, T., Sidman, R.L., Glimcher, M.J., and Glimcher, L.H.:**  
Chondrodysplasia and neurological abnormalities in ATF-2-deficient mice.  
Nature 379 (1996), 262-265.

**Reznikoff, C. A. *et al*:** Establishment and Characterization of a Cloned Line of CH3 Mouse Embryo Cells Sensitive to Postconfluent Inhibition of Division. Cancer Res. 33 (1973), 3231-3238.

**Roberts, A.B., Anzano, M.A., Lamb, L.C., Smith, J.M., and Sporn, M.B.:** New class of transforming growth factors potentiated by epidermal growth factor: isolation from nonneoplastic tissues. Proc. Natl. Acad. Sci. USA 78 (1981), 5339-5343.

**Roberts, A.B., and Sporn, M.B.:** The transforming growth factor  $\beta$ s. In : Peptides, Growth factors and Their receptors, Part I, pp 419-472 , 1990, Sporn M.B., and Roberts, A.B. (eds.) Springer-Verlag, Berlin.

**Rosen, V., Cox, K., Hattersley, G., *et al*.**: Bone morphogenetic proteins. In Iliopoulou JP, Raisz, JG, Rodan, GA (eds). Principles of Bone Biology. New York, Academic Press 661-671, 1996.

**Sampath, T.K., Maliakal, J.C., Hauschka, P.V., *et al*.** Recombinant human osteogenic protein-1 (hOP-1) induces new bone formation in vivo with a specific activity comparable with natural bovine osteogenic protein and stimulates osteoblast proliferation and differentiation in vitro. J. Biol.Chem. 267 (1992), 20352-20362.

**Sanger, F., Nicklen, S., and Coulson, A.R.:** DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. 74 (1977), 5463-5467.



**Savage, C., Das, P., Finelli, A., Townsend, S., Sun, C., Baird, S., and Padgett, R.:**

Caenorhabditis elegans genes sma-2, sma-3 and sma-4 define a conserved family of transforming growth factor  $\beta$  pathway components. Proc. Natl. Acad. Sci. USA 93 (1996), 790-794.

**Schüle, R., Umesono, K., Mangelsdorf, D.J., Bolado, J., Pike, J.W., and Evans, R.M.:**

Jun-Fos and receptors for Vitamin A and D recognize a common response element in the human osteocalcin gene. Cell 61 (1990), 497-504.

**Shi, Y.G., Hata, A., Lo, R.S., Massagué, J., and Pavletich, N.P.:** A structural basis for mutational inactivation of the tumor suppressor Smad4. Nature 388 (1997), 87-92.

**Shibuya, H., Yamaguchi, K., Shirakabe, K., Tonegawa, A., Gotoh, Y., Ueno, N., Irie, N., Nishida, E., and Matsumoto, K.:**

TAB1: an activator of TAK1 MAPKKK in TGF- $\beta$  signal transduction. Science 272 (1996), 1179-1182.

**Souchelnytskyi, S. Tamaki, K., Engström, U., Wernstedt, C., ten Dijke, P., and Heldin, C-H.:**

Phosphorylation of Ser465 and Ser467 in the C-terminus of Smad2 mediates interaction with Smad4 and is required for TGF- $\beta$  signaling. J. Biol. Chem. 272 (1997), 28107-28115.

**Southern, P.J., and Berg, P.:** Transformation of mamalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Gen. 1(1982), 327-341.

**Suzuki, A., Ueno, N., and Hemmati-brivanlou, A.:** Xenopus msx1 mediates epidermal induction and neural inhibition by BMP4. development 124 (1997), 3037-3044.

**Takase, M., Imamura, T., Sampath, T.K., Takeda, K., Ichijo, H., Miyazono, K., and Kawabata, M.:**

Induction of Smad6 mRNA by bone morphogenetic proteins. *Biochem. Biophys. Res. Commun.* 244 (1998), 26-29.

**Takuwa, Y., Ohse, C., Wang, E.A., Wozney, J.M., and Yamashita, K.:** Bone morphogenetic protein-2 stimulates alkaline phosphatase activity and collagen synthesis in cultured osteoblastic cells, MC3T3-E1. *Biochem. Biophys. Res. Commun.* 174 (1991), 96-101.

**Ten Dijke, P., Miyazono, K., and Heldin C-H.:** signaling via hetero-oligomeric complexes of type I and type II receptors. *Curr. Opin. Cell. Biol.* 8 (1996), 139-145.

**Ten Dijke, P., Yamashita, H., Sampath, T.K., Reddi, A.H., Estevez, M., Riddle, D.L., Ichijo, H., Heldin, C-H., and Miyazono, K.:** Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4. *J. Biol. Chem.* 269 (1994), 16985-16988.

**Termine, J.D., Kleinmann, H.K., Whitson, S.W., Conn, K.M., McGarvey, M.L., and Martin, G.R.:** Osteonectin, a bone specific protein linking mineral to collagen. *Cell* 26 (1981), 99-105.

**Thies, R.S., Bauduy, M., Ashton, B.A., Kurtzberg, L., Wozney, J.M., and Rosen, V.:** Recombinant human bone morphogenetic protein-2 induces osteoblastic differentiation in W-2017 stromal cells. *Endocrinology* 130 (1992), 1318-1324.

**Thompson, J.F., Hayes, L.S., Lloyd, D.B.:** Modulation of firefly luciferase stability and impact on studies of gene regulation. *Gene* 103 (1993), 171-177.

**Tokuwa, Y., Ohse, C., Wang, E.A., Wozney, J.M., and Yamashita, K.:** Bone morphogenetic protein-2 stimulates alkaline phosphatase activity and collagen

- synthesis in cultured osteoblastic cells, MC3T3-E1. *Biochem. Biophys. Res. Commun.* 174 (1991), 96-101.
- Tracy, R.P., Shull, S., Riggs, B.L., and Mann, K.G.:** The osteonectin family of proteins. *Int. J. Biochem.* 20 (1988), 653-660.
- Tsuji, K., Ito, Y., and Noda, M.:** Expression of the PEBP2aA/AML3/CBFA1 gene is regulated by BMP4/7 heterodimer and its overexpression suppressed type I collagen and osteocalcin gene expression in osteoblastic and nonosteoblastic mesenchymal cells. *Bone* 22 (1998), 87-92.
- Taylor, S.M., and Jones, :** Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-Azacytidine. *Cell* 17 (1979), 771-779.
- Urena, P., Kong, X-F., Abou-Samra, A-B., Jüppner, H., Kronenberg, H.M., Potts, J.T., JR, and Segre, G.V.:**  
Parathyroid Hormone (PTH)/PTH-related peptide receptor messenger ribonucleic acids are widely distributed in rat tissues. *Endocrinology* 133 (1993), 617-623.
- Vainio, S., Karavanova, I., Jowett, A., and Thesleff, I.:** Identification of BMP4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development. *Cell* 75 (1993), 45-58.
- Vortkamp, A., Lee, K., Lanske, B., et al.:** Regulation of rate of cartilage differentiation by Indian hedgehog and parathyroid hormone related peptide. *Science* 273 (1996), 579-582.
- Wang, E.A., Rosen, V., D'Alessandro, J.S., et al.:** Recombinant human bone morphogenetic protein induces bone formation. *Proc. Natl. Acad. Sci. USA.* 87 (1990), 2220-2224.

- Wang, E.A., Israel, D.I., Kelly, S., and Luxenberg, D.P.:** Bone morphogenetic protein-2 causes commitment and differentiation in C3H10T1/2 and 3T3 cells. *Growth Factors* 9 (1993), 57-71.
- Wang, T., Donahoe, P.K., and Zervos, A.S.:** Specific interaction of type I receptors of the TGF-beta family with the immunophilin FKBP-12. *Science* 265 (1994), 674-676.
- Wantanable, T.K., Suzuki, M., Omori, Y., Hishigaki, H., Horie, M., Kanemoto, N., Fujiwara, T., Nakamura, Y., and Takahashi, E-I. :**  
Cloning and characterization of a novel member of human Mad gene family. *Genomics* 42 (1997), 446-451.
- Weis-Garcia, F. and Massagué, J.:** Complementation between kinase-defective and activation-defective TGF- $\beta$  receptors reveals a novel form of receptor cooperativity essential for signaling. *EMBO J.* 15 (1996), 276-289.
- Wharton, K.A., Thomsen, G.H., and Gelbart, W.M.:** *Drosophila* 60 A gene, another transforming growth factor beta family member, is closely related to human bone morphogenetic proteins. *Proc. Natl. Acad. Sci.* 88 (1991), 9214-9218.
- Wiersdoff, V., Lecuot, T., Cohen, S.M., and Mlodzik, M.:** *Mad* acts downstream of Dpp receptors, revealing differential requirement for *dpp* signaling in initiation and propagation of morphogenesis in the *Drosophila* eye. *Development* 122 (1996), 2153-2162.
- Wozney, J.M.:** Bone morphogenetic proteins. *Prog. Growth Factor Res.* 1 (1989), 267-280.
- Wozney, J.M., and Rosen, V.:** Bone morphogenetic proteins. *Handbook Exp. Pharmacol.* 107 (1993), 725-748.

**Wozney, J.M., and Rosen, V.:**

Bone Morphogenetic Protein and Bone Morphogenetic Protein Gene Family in Bone Formation and Repair. *Clinical Orthopaedics and Research* 346 (1998), 26-37.

**Wozney, J.M., Rosen, V., Celeste, A.J., Mistsock, L.M., Whitters, M.J., et al.:**

Novel regulators of bone formation: molecular clones and activities. *Science* 242 (1988), 1528-1534.

**Wrana, J.L., and Attisano, L.:** MAD-related proteins in TGF $\beta$  signaling. *Trends Genet* 12 (1996), 493-496.

**Wrana, J.L. and Pawson, T.:** Mad about SMADs. *Nature* 388 (1997a), 28-29.

**Wrana, J.L., Attisano, L., Wieser, R., Ventura, F., and Massagué, J.:**

Mechanism of activation of TGF- $\beta$  receptor. *Nature* 370 (1994a), 341-347.

**Wrana, J.L., Attisan, L., Cárcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, W-F., and Massagué, J.:**

TGF $\beta$  signals through a heteromeric protein kinase receptor complex. *Cell* 71 (1992), 1003-1014.

**Wrana, J.L., Tran, H., Attisano, L. et al :**

Two distinct membrane serine/threonine kinase from *Drosophila* from an activin receptor complex. *Mol. Cell. Biol.* 14 (1994b), 944-950

**Yamaguchi, A., Katagiri, T., Ikeda, T., Wozney, J.M., Rosen, V., Wang, E.A., Kahn, A.J., Suda, T., and Yoshiki, S.:**

Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation *in vitro*. *J. Cell. Biol.* 113 (1991), 681-687.

**Yamaguchi, K., Shirakabe, K., Shibuya, H., Irie, K., Oishi, I., Ueno, N., Taniguchi, T., Nishida, E., and Matsumoto, K.:**

Identification of a member of the MAPKKK family as a potential mediator of TGF- $\beta$  signal transduction. *Science* 270 (1995), 2008-2011.

**Yamashita, H., ten Dijke, P., Franzen, P., Miyazono, K., and Heldin, C-H.:** *J. Biol. Chem.* 269 (1994), 20172-20178.

**Yingling, J.M., Das, P., Savage, C., Zhang, M., Padgett, R.W., and Wang, X-F.:**

Mammalian dwarfins are phosphorylated in response to TGF- $\beta$  and are implicated control of cell growth. *Proc. Natl. Acad. Sci.* 93 (1996), 8940-8944.

**Yingling J.M., Datto, M.B., Wong, C., Frederick, J.P., Liberati, N.T., and Wang, X-F.:**

The tumor suppressor, Smad4, is a TGF- $\beta$  inducible, DNA binding protein. *Mol. Cell. Biol.* 17 (1997), 7019-7028.

**Young, D.C., Kingsley, S.D., Ryan, K.A., and Dutko, F.J.:** Selective inactivation of eukaryotic beta-galactosidase in assays for inhibitors of HIV-1 TAT using bacterial beta-galactosidase as a reporter enzyme. *Anal. Biochem.* 215 (1993), 24

**Zawel, L., Dai, J.L., Buckhaults, P., Zhou, S., Kinzler, K.W., Vogelstein, B., and Kern, S.E.:**

Human Smad3 and Smad4 are sequence-specific transcription activators. *Mol. Cell* 1 (1998), 611-617.

**Zhang, Y., Musci, T., and Derynck, R.:** The tumor suppressor Smad4/DPC4 as a central mediator of Smad function. *Current Biology* 7 (1997), 270-276.

**Zhang, Y., Feng, X-H., Wu, R-Y., Derynck, R.:** Receptor-associated Mad homologues synergize as effectors of the TGF- $\beta$  response. *Nature* 383 (1996), 168-172.

**Zhao, G-Q., Deng, K., Labosky, P.A., Liaw, L., and Hogan, B.L.M.:** The gene encoding bone morphogenetic protein 8B (BMP8B) is required for the initiation and maintenance of spermatogenesis in the mouse. *Genes and Development* 10 (1996),

**Zhou, X., Sasaki, H., Lowe, L., Hogan, B.L.M., and kuehn, M.R.:** Nodal is a novel TGF $\beta$ -like gene expressed in the mouse node during gastrulation. *Nature* 361 (1993) 543-547.

**Zou, H., and Niswander, L.:** Requirement for BMP signaling in interdigital apoptosis and scale formation. *Science* 272 (1996), 738-741.

## 7 Appendix

## 7.1 Oligonucleotide

Primer	Sequence (5'→ 3')	Annealing temp.	Fragment length	Notes
<b>T7 Primer 5'</b>	TAGTCTTTTGTCTTTTAT	45°C		pMT7T3 5' primer
<b>T3 Primer 3'</b>	AGCTCAATTAACCCTCACT AAAGGGAGGTACCA	45°C		pMT7T3 3' primer
<b>P-Smad3 5'</b>	TCGAATTCGCCACCATGAT CCCGGCCGAGTTCCCCC ACT	50°C	276 bp	cloning 5' primer of Smad3 P domain
<b>P-Smad3 3'</b>	ATCGAAGCTTTTAACCGTA GGTGACTGGCTGTAG	50°C	276 bp	cloning 5' primer of Smad3 P domain
<b>RT-AdipoQ 5'</b>	GCAACATTCCGGGACTCTA CTACT	54,7°C	541 bp	MUR 5'
<b>RT-AdipoQ 3'</b>	ATTGTGAAGCCCCATACC A	54,7°C	541 bp	MUR 3'
<b>RT-BMP-2 5'</b>	GTTTGTGTTTGGCTTGACG C	50°C	720 bp	bone morphogenetic protein 2; Wiles, 1995
<b>RT-BMP-2 3'</b>	AGACGTCTCAGCGAATTT G	50°C	720 bp	bone morphogenetic protein 2; Wiles, 1995
<b>OSF2/CBFA1 2 5'</b>	TCCTACCAGCCACCGAG ACCAA	54,8°C	127 bp	MUR
<b>OSF2/CBFA1 2 3'</b>	CACTGCGCTGAAGAGGCT GTTT	54.8°C	127 bp	MUR
<b>RT-CBFA1 5'</b>	CAGGAAGACTGCAAGAAG GCTCTGG	57°C	186 bp	MUR 5'
<b>RT-CBFA1 3'</b>	ACACGGTGTCCTGCGCT GAAGA	57°C	186 bp	MUR 3'
<b>RT-CollagenI 5'</b>	GCCCTGCCTGCTTCGTG	50°C	252 bp	MUR
<b>RT-CollagenI 3'</b>	CGTAAGTTGGAATGGTTTT T	50°C	252 bp	MUR
<b>RT-Collagen II 5'</b>	CCTGTCTGCTTCTGTAAA AC	54°C	342 bp	MUR
<b>RT-Collagen II 3'</b>	AGCATCTGTAGGGGTCTT CT	54°C	342 bp	MUR
<b>RT-HPRT 5'</b>	GCTGGTGAAAAGGACCTC T	50-55°C	249 bp	Hypoxanthin-Guanin-Phosphori bosyl-Transferase; Wiles, 1995
<b>RT-HPRT 3'</b>	CACAGGACTAGAACACCT GC	50-55°C	249 bp	Hypoxanthin-Guanin-Phosphori bosyl-Transferase; Wiles, 1995



Primer	Sequence (5' → 3')	Annealing temp.	Fragment length	Notes
RT-Smad1 3'	GCTGGTTGGGGAGTGAGG GTAGGT	59°C	531 bp	MUR
RT-Smad1 5'	GCTGTTGACGCTTTGGTG AA	59°C	531 bp	MUR
RT-Smad2 5'	TGTTTGCTGAGTGCCTAAG TGATA	55.2°C	472 bp	MUR
RT-Smad2 3'	AAGTGCTGTTTTTGTCTG GA	55.2°C	472 bp	MUR
RT-Smad3 5'	CAGTGCTATTTTCGTCCAG TCTCC	58.7°C	836 bp	MUR
RT-Smad3 3'	CCAGCTCTACCTCCGGC GTGTTT	58.7°C	836 bp	MUR
RT-Smad4 5'	CTTCGGGAGGAGATCGCT TTTG	60.2°C	502 bp	DPC4 (MUR)
RT-Smad4 3'	CACGGGGTTTCCTTGATG CTCT	60.2°C	502 bp	DPC4 (MUR)
RT-Smad6 5'	GCCCGCTCGCGACTGCTG CTTCT	64.5°C	745 bp	MUR
RT-Smad6 3'	GGTGCTCGCCCCGGTTGT AGG	64.5°C	745 bp	MUR
RT-Smad7 5'	TCGCGTGGGGAGGCTCTA CTGTGT	59.6°C	469 bp	MUR
RT-Smad7 3'	CTACCGCTGTTGAAGAT GACCTC	59.6°C	469 bp	MUR
RT-Smad1 5' xen	GAAATGGGCAGAGAAAGC AGTAGA	57,6	1310 bp	xenopus
RT-Smad1 3' xen	AGAGACCGAGGAGATGGG ATTATG	576	1310 bp	xenopus
RT-CHMAD 5'	ACTGTGTCACCATTCCTCCG CTCTC	55°C	589 bp	human, Canada
RT-CHMAD 3'	TCTCCTCTGTTGATTTCTG A	55°C	589 bp	human, Canada
RT-XSmad1 NP 5'	ACCAGCCCAGCAGTGAAG AGG	58.2°C	443 bp	xenopus(N+P)
RT-XSmad1 NP 3'	CGGAATTGCGCAAGGAGA CTGT	58.2°C	443 bp	xenopus(N+P)
RT-XSmad1 C 5'	CTATGTTGGGGTTGAAG TCTAT	53.8°C	320 bp	xenopus C
RT-XSmad1 C 3'	GGGGGCCGTGCAGGTGAA T	53.8°C	320 bp	xenopus C
RT-XSmad1 N 5'	AGAGGCTGCTTGTTGG	54°C	208 bp	xenopus N
RT-XSmad1 N 3'	GGTAGGCCCTTGCGGTGT GA	54°C	208 bp	xenopus N

Primer	Sequence (5'→ 3')	Annealing temp.	Fragment length	Notes
RT-XSmad1 P 5'	GCCTCACAACGCAACTTTT C	55°C	285 bp	xenopus P
RT-XSmad1 P 3'	GCCTGGACATCTGCTCTAT T	55°C	285 bp	xenopus P
RT-XSmad1(del 1-144)	GCCTCACAACGCAACTTTT C	56.1°C		xenopus(C+P) 5'
RT-Osteocalcin 5'	GCAGACCTAGCAGACACC AT	57°C	419 bp	MUR
RT-Osteocalcin 3'	GAGCTGCTGTGACATCCAT AC	57°C	419 bp	MUR
RT-Osteonectin 5'	CCCTCTGCGCATCGGTGA CT	59.7°C	488 bp	MUR
RT-Osteonectin 3'	GGGGGTAATGGGAGGGGT GAC	59.7°C	488 bp	MUR
RT-Osteopontin 5'	ACATCAGAGCCACGAGTTT CACA	53°C	423 bp	MUR
RT-Osteopontin 3'	TTTGCCTGCCTCTACATAC ATTTT	53°C	423 bp	MUR
RT-pCS2 5'	CCCAACTTGATTTAGGTGA C			pCS2 5' primer
RT-pCS2 3'	AATACGACTCACTATAG			pCS2 3' primer
RT-PTH/PTHrP R 5'	GTTGCCATCATATACTGTT TCTGC	59°C	473 bp	MUR
RT-PTH/PTHrP R 3'	GGCTTCTTGGTCCATCTGT CC	59°C	473 bp	MUR

**Notes:** All the primers were purchased from firm Gibco.

## 7.2 Plasmids

Plasmids	Function	Source of supply
pAG 60	used as a selection plasmid in permanent transfections	Promega(Colbe're-Garapin, 1981)
pBSpAC <sup>p</sup>	used as a selection plasmid in permanent transfections	Kirchhoff, S. (Genetic, GBF, Braunschweig)
pGL2-Basic	Luciferase expression plasmid lacks eukaryotic promoter and enhancer sequences. The JunB promoter was cloned in this vector.	Promega
pMT7T3	used as an eukaryotic expression plasmid	Dirks, W. (Genetic, GBF, Braunschweig) (Ahrens et al. 1993)

## 7.3 Materials and Instruments

### 7.3.1 Instruments

autoclave	Westerma Sauter LAF
cell culture documentation:	INTAS
cell culture incubator:	Heraeus B 5061 EK/02
cool centrifuge:	Sorvall Superspeed RC5-C with roter GS3, GSA, SS34 and HB-4
electrophoresis apparatus:	Biometra, PP2000
electrotransformation apparatus:	Bio-Rad Gene Pulser
gel documentation:	PHASE, GelCam
lumat (for Luciferase) LB 9501:	Berthold
microlumat (for $\beta$ -Gal) LB 96 P:	EG & G Berthold
microtiterplate(MTP) white:	EG & G Berthold
microscope:	Leitz, Labovert FS
PCR apparatus:	Perkin Elmer, Gene AMP PCR System 2400
pH meter:	50 Beckman
photo meter:	Spectronic, Genesys 2; Pharmacia, GeneQuant
shaking appartus:	Heidorf Reax 2000
sequence apparatus:	Pharmacia LKB-ALF DNA Sequencer ABI Prism 310 Genetic Analyzer
sterile bank:	Heraeus Lamin Air
table centrifuge:	Heraeus Christ Biofuge A Eppendorf 5417 R
thermomixer:	Eppendorf Thermomixer 5436
vacuum centrifuge:	Savant Speedvac Concentrator
waterbath:	Haake D8-L, GFL model 1004 (Kötter)

### 7.3.2 Materials and cell culture media

sterile filter:	Sartorius membrane filter; Pore size 0,22 $\mu\text{m}$
cell culture flasks:	Nunc
FCS:	CTM, Chemie-Technik-München
DME:	GIBCO BRL

The other materials and instruments were standard laboratory equipments and had no relative effects on documentations of experiments.

### 7.3.3 Enzymes and chemicals

The restriction endonucleases, alkaline phosphatase (Shrimp), T4-Polynucleotide kinase, Klenow-Fragment, M-MLV-Reverse Transcriptase and Taq-DNA-Polymerase were supplied by firm Biolabs, Boehringer, GIBCO BRL, Pharmacia, Promega and USB.

Chemicals were purchased from Bayer, Biolabs, Bio-Rad, Boehringer, Fluka, GIBCO BRL, Merck, Pharmacia, Renner, Riedel-de-Haen, Roth, Seromed, Serva and Sigma.

### 7.3.4 Computerprograms/Data Banks

Adobe Photoshop 3.0 / 4.0

CorelDraw 7.0

DNASTAR 1.0

Excel 5.0

Medline

MVPilot 1.0 Zellkulturdokumentation

Vector NTI

WinCam 1.0

WIN GLOW

Lotus SmartSuite 97- Word Pro

## 7.4 Abbreviations

<b>A</b>	adenosine
<b>Amp</b>	ampicillin
<b>APS</b>	ammonium peroxydisulfate
<b>ATP</b>	adenosine triphosphate
<b><i>Bam</i>HI</b>	<b>a</b> restriction endonuclease
<b>bFGF</b>	basic fibroblast growth factor
<b>BMP</b>	bone morphogenetic protein
<b>BSA</b>	bull serum albumin
<b>bp</b>	base pair
<b>β-Gal</b>	β-galactosidase
<b>cDNA</b>	complementary DNA
<b>dATP</b>	desoxyadenosin triphosphate
<b>dCTP</b>	desoxycytidin triphosphate
<b>ddATP</b>	dideoxyadenosin triphosphate
<b>ddCTP</b>	dideoxycytidin triphosphate
<b>ddGTP</b>	dideoxyguanosin triphosphate
<b>ddTTP</b>	dideoxythymidin triphosphate
<b>DEPC</b>	diethylpyrocarbonate
<b>dGTP</b>	desoxyguanosin triphosphate
<b>DMSO</b>	dimethylsulfoxid
<b>DNA</b>	desoxyribose nucleic acid
<b>dNTP</b>	desoxynucleotid triphosphate
<b>dsDNA</b>	double-stranded DNA
<b>DTT</b>	dithiothreitol
<b>dTTP</b>	desoxythymidin triphosphate
<b><i>E. coli</i></b>	<i>Escherichia coli</i>
<b><i>E. coli</i> SURE</b>	<i>E. coli</i> ‘Stop Unwanted Rearrangement Events’
<b>EDTA</b>	ethylendiaminetetraactetic acid
<b>EGF</b>	epidermal growth factor

<b>Erk</b>	extracellular stimulus responsive kinase
<b>EtBr</b>	ethidium bromid
<b>EtOH</b>	ethanol
<b>FCS</b>	fetal calf serum
<b>g</b>	gram
<b>hr</b>	hour
<b>HEPEs</b>	N-2-Hydroxyethylpiperazin-N'-2-ethanesulfonic acid
<b><i>HindIII</i></b>	a restriction endonuclease
<b>kb</b>	1000 base pair
<b>Kv</b>	kilovolt
<b>l</b>	liter
<b>LMP</b>	low melting point
<b>M</b>	molar concentration
<b>mA</b>	milliampère
<b>Mad</b>	mothers against <i>dpp</i>
<b>mg</b>	milligram
<b>min</b>	minute
<b>ml</b>	milliliter
<b>mM</b>	millimol
<b>mRNA</b>	messenger RNA
<b><math>\mu</math></b>	micro
<b><math>\mu</math>g</b>	microgram
<b><math>\mu</math>l</b>	microliter
<b><math>\mu</math>m</b>	micrometer
<b>NaAc</b>	sodium acetate
<b>ng</b>	nanogramm
<b>nm</b>	nanometer
<b>nmol</b>	nanomol
<b>OD</b>	optical density
<b>PCR</b>	polymerase chain reaction
<b>RNA</b>	ribonucleic acid
<b>RNase</b>	ribonuclease

<b>RNasin</b>	RNase inhibitor
<b>rpm</b>	rounds per minute
<b>RT</b>	room temperature
<b>RT-PCR</b>	reverse transcription polymerase chain reaction
<b>SDS</b>	sodium dodecyl sulfate
<b>sec</b>	second
<b>ss</b>	single stranded
<b>T</b>	thymidine
<b>TE</b>	Tris-EDTA
<b>TEMED</b>	N, N, N', N'-tetramethyl ethylene diamine
<b>TGF</b>	transforming growth factor
<b>U</b>	unit
<b>UV</b>	ultraviolet
<b>Vol</b>	volume
<b>w/v</b>	weight per volume



